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SUBSPECIATION IN *TRIFOLIUM SUBTERRANEUM*

By F. H. W. Morley,* R. D. Brock,* and C. I. Davern*

[*Manuscript received August* 10, 1955]

Summary

Subspeciation in *Trifolium subterraneum* was indicated by the presence of reproductive and vegetative abnormalities in crosses between some varieties.

In F_1's and F_2's there was pollen sterility, reduced seed set, and embryo abortion. Dwarf and semi-dwarf plants segregated in some F_2's. The occurrence and severity of different abnormalities were strongly correlated between F_2's. Within F_2's the correlation was slight or absent which suggested that these abnormalities were independently determined. Chromosomal rearrangement was indicated by failure of pairing, reduced chiasma frequency, and multivalent formation at meiosis of some F_1's. Less than 40 per cent. of observed pollen sterility could be explained by such structural differences. The main cause of sterility seems to be genic.

The presence of genic and structural sterility barriers suggests that many of the varieties were reproductively isolated long before they were introduced to Australia. Major varietal differences are unlikely to have arisen from selection of locally adapted ecotypes. There is thus ample justification for a programme of hybridization and selection to improve and to extend the range of the species. Such a programme would be impeded, but not prevented, by the presence of reduced fertility in some segregates.

I. Introduction

Subterranean clover (*Trifolium subterraneum* L.) consists of numerous self-pollinating varieties showing a wide morphological and physiological diversity. The natural habitat of the species is the Mediterranean region, the Iberian peninsula, France, and the British Isles. Strains from Portugal, Morocco, Malta, England, and commercial Australian varieties have $2n = 16$ chromosomes. A "strain" from Israel has $2n = 12$ chromosomes (Yates and Brittan 1952).

In Australia the species is distributed mainly in southern areas with 20 in. or more annual precipitation. Collections made in this area were described by Aitken and Drake (1941) who recorded over 40 distinct types. According to these authors, and to Donald and Smith (1937) and Frankel (1955) these were probably introduced independently, either deliberately or accidentally. Davies (1951) considered that some introductions came from England and not from the Mediterranean.

The existence of a cryptic species in Israel (Brock 1953), and the variable success of intervarietal crosses (Hutton and Peak 1954) suggested that differences of subspecific rank may distinguish some Australian varieties. Accordingly various characteristics of parent and hybrid plants were examined. The results support the view that some strains are differentiated by genic and chromosomal sterility barriers. Such differences indicate long term genetic separation and, we consider, constitute the process of subspeciation such as described in other genera by Stebbins (1950), Baker (1951), Clausen (1951), and others.

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TABLE 1

DISTRIBUTION OF POLLEN STERILITY IN PARENTS AND CROSSES

Numbers of plants showing different pollen scores

Parents and Crosses	Plants	Total Plants	Mean Pollen Score										Mean
			5	15	25	35	45	55	65	75	85	95	
Parents	Tallarook	5	5										5
	458	35	31	2			1					1	6
	Clare	3	3										5
	Mulwala	5	4	1									7
	Gin Gin	4	4										5
	Red Leaf	4	4										5
	Mt. Barker	5	4				1						13
	Dalliak	4	3	1									7
	Yarloop	5	1	1	1	1		1					27
	Dwalganup	4	4										5
	N_1E	5	5										5
	Pink Flower	5	2	3									11
F_1's	Burnerang × Wenigup*	2									2		85
	Tallarook × Clare*	2								1	1		80
	,, × Wenigup*	2								1	1		80
	,, × 458	7							5	2			68
	,, × Dwalganup	19			4	5	8			2			41
	,, × Pink Flower	4				2	2						40
	,, × Red Leaf	3		2		1							22
	,, × Gin Gin	9	9										5
	,, × Mt. Barker	12	12										5
	Mt. Barker × Dwalganup	15		4	6	3	1			1			29
	Pink Flower × Red Leaf*	1		1									15
	Yarloop × Yabba North	2		2									15
F_2's	Tallarook × Clare	65	5	3	1		9	8	14	12	10	3	60
	,, × Yarloop	20	1	3		1	1	3	3	2	1	5†	60
	,, × 458	108	21	12	12	7	13	11	10	3	2	17†	43
	,, × Dwalganup	86	33	15	12	12	8	2	3	1			22
	,, × Pink Flower	39	17	12	2	3	1		1	2	1		20
	,, × Mulwala	24	11	7	1		3	1		1			20
	,, × N_1E	23	16	3		3				1			13
	,, × Cranmore	13	8	2	2	1							13
	,, × Dalliak	47	34	8	5								9
	,, × Red Leaf	5	5										5
	,, × Gin Gin	8	8										5
	,, × Mt. Barker	23	22						1				8
	Mt. Barker × Pink Flower	33	14	4	4	4	3	4					22
	,, × Dwalganup	56	24	16	5	4	1	2	2		1	1	19
	,, × N_2E	20	18		1	1							7
	N_1E × Pink Flower	22	11	3	3	1	1	2	1				20
	,, × N_2E	14	12	1			1						9
	,, × Dwalganup	23	19	4									7
	,, × Mt. Barker	23	21	2									6
	,, × Cranmore	10	9	1									6
	Clare × Pink Flower	24	9	8	1	1	2	1	1	1			21

* Plants grown in glasshouse, remainder in field.

† These plants classified "empty anthers".

TABLE 1 (*Continued*)

Parents and Crosses	Plants	Total Plants	Mean Pollen Score										Mean
			5	15	25	35	45	55	65	75	85	95	
Backcross to Tallarook of F_2's of:	Dwalganup*	2		2									15
	Yarloop*	1							1				65
	N_1E*	1	1										5
	Gin Gin*	3	3										5

* Plants grown in glasshouse, remainder in field.

II. METHODS AND MATERIAL

Most of the plants studied (see Table 1) were grown at Dickson Experiment Station, Canberra. Seedlings were grown in flats and transplanted to the field in May 1954, spaced 7 lk each way between plants. Spray irrigation was provided when necessary. The original crosses were made in connection with the programme of Dr. E. M. Hutton in his investigations of inheritance in this species. Some additional material was provided from crosses made by the senior author in the spring of 1954, and grown in the glasshouse during the summer 1954–1955.

(*a*) *Pollen Sterility*

Pollen sterility was assessed by the reaction of grains to staining with cotton blue in lactophenol after fixation in acetic alcohol (Darlington and La Cour 1947). During November 1954 two inflorescences, just prior to anthesis, were collected from five plants of each parent variety, from all F_1's, and from at least 25 plants of most F_2 lines.

Because of variability between anthers and flowers and the large amount of material involved, a rapid rating for pollen sterility was used. Slides were graded 0–10, 10–20, . . . , 90–100 estimated percentage of abnormal pollen, using 5, 15, . . . , 95 for calculating means. This scoring system was found to be satisfactory and it gave good agreement with actual counts.

(*b*) *Seed Set*

Seed set was examined in (1) a sample of 10 well-developed burrs taken during late November from each of four to five plants, and (2) whole runners collected during the first week in December. These were stored by refrigeration for examination during the subsequent 3 weeks. Seeds were classified as follows:

(i) *Normal*.—Seeds with fully distended seed coats, apparently viable (but not necessarily large).

(ii) *Shrivelled*.—Slightly to very shrivelled seeds, with incompletely developed embryos. These were considered to correspond to the aborted seeds noted in many interspecific crosses (Brink and Cooper 1947).

(iii) *Empty Flowers*.—No seed development, indicating failure of fertilization or very early embryo degeneration.

(c) *Meiosis*

Meiosis was studied in acetic orcein squashes of pollen mother cells after fixation in Carnoy fluid (6:3:1) (Darlington and La Cour 1947).

III. Results

(a) *Pollen Sterility*

Table 1 shows the pollen scores of parental, F_1, and F_2 lines in terms of number of plants in each class. An additional class of "empty anthers" gives the number of plants in which no organization of pollen grains was noted in all anthers examined (usually 8-10). Occasional loose pollen grains were seen in a few of these slides. These plants were allocated a value of 95 when compiling means.

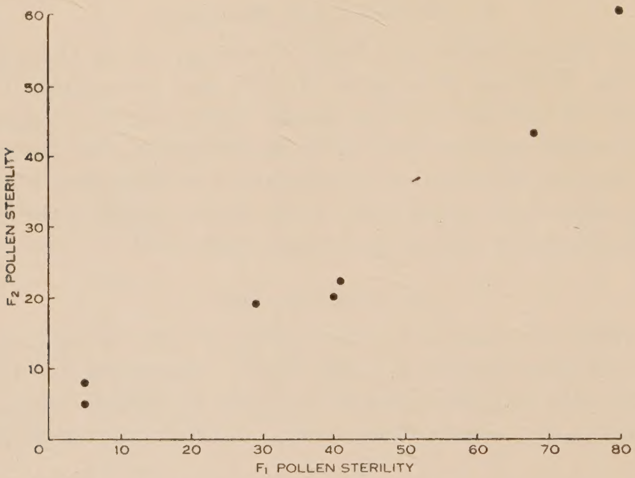


Fig. 1.—Correlation between pollen sterility in F_1 and F_2.

The data of Table 1 indicate that, with few exceptions, plants in the parental lines were highly fertile. There is relatively little variation in pollen score among plants in an F_1, but there is a wide range between different F_1's. There is a wide variation within and between F_2's, indicating segregation for this characteristic. F_1 lines of high pollen sterility segregated high and low sterility plants in F_2. However, fully fertile F_1's showed no segregation for pollen sterility in F_2.

The correlation between pollen score in F_1 and F_2 is shown in Figure 1. The correlation is high, especially in view of the small number of plants scored in F_1. In general, the F_2's were more fertile than F_1's, presumably because many unfavourable genotypes were eliminated in F_1.

Glasshouse material was collected from plants grown during January-March 1955 under a 16-hr day (part of period only) after 3 weeks vernalization. Pollen scores indicate a strong incompatibility between Tallarook and Wenigup, Burnerang and Wenigup, and Tallarook and Clare. The last observation is in accord with field observations, and the pollen scores of backcrosses suggest that environment conditions were not unfavourable to normal pollen formation.

The plants with empty anthers in the crosses Tallarook × 458 F_2 and Tallarook × Yarloop F_2 are of particular interest. The anthers were approximately normal in size, and appeared distended with cellular material, but no organization of pollen grains was apparent (Plate 1). It is tempting to suggest that this abnormality is determined by a recessive gene which is segregating in a 3:1 ratio in these F_2's. In view of the breeding habit of the parent varieties such an explanation is very unlikely, as one parent would be homozygous for the gene in question. A 13:3 ratio is consistent with the data. Nearly all these empty anther plants set a small quantity of seed, so that breeding tests will be possible. An occasional apparently normal pollen grain was noted in some preparations. In one plant which set no seeds, no pollen grains were found in 93 anthers examined.

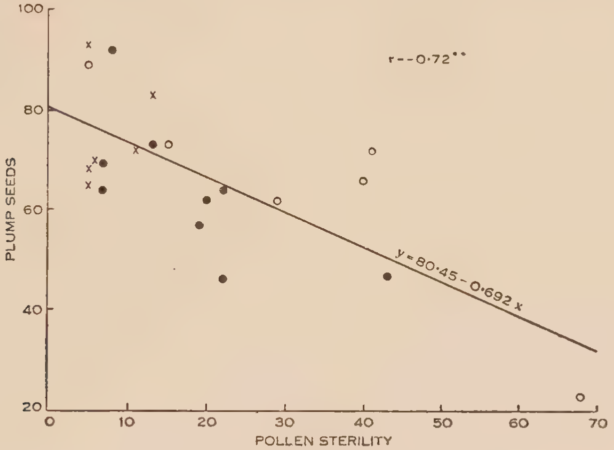


Fig. 2.—Relationship between pollen sterility (Table 1) and formation of plump seeds (Table 2). × Parents. ○ F_1's. ● F_2's.

In addition to the plants with empty anthers there were plants in which some sectors of some anthers examined contained little or no pollen. There were a few obvious variations between anthers of the same flower or different flowers on the same or different inflorescences. Such variation was not noted in any preparation from parent lines, except in the variety Yarloop. The significance of these variations will be discussed in Section IV of this paper.

(*b*) *Seed Set*

Seed set as indicated in Section II (*b*) was determined from burr samples and on whole runners. Whole runners give an indication of the ability to set seed under the particular set of external and internal environmental conditions over a period of time. These conditions will also vary throughout the field, so that comparisons between groups are not strictly valid, because there will be differences due to maturity, onset of senescence, and disease.

If p were the inherent fertility of the plant, then the probability of any of the four flowers which normally constitute an inflorescence failing to set and develop seed would be $(1-p)^4$. The sample of 10 burrs per plant would exclude the $(1-p)^4$

inflorescences which failed completely, but would include inflorescences in which one or more seeds developed, as burr formation is normal in these cases. However, the frequency of inflorescences failing to produce burrs was considerably higher than expected. This is not surprising since all flowers of an inflorescence would be influenced by the same environment, and would tend to succeed or fail together.

TABLE 2

PERCENTAGE OF THE DIFFERENT CATEGORIES OF SEEDS IN PARENTS AND HYBRIDS

Parents and Crosses	Plants	No. of Plants Sampled	Total Flowers	Plump Seeds (%)	Shrivelled Seeds (%)	Empty Flowers (%)	Seed Abortion (%)
Parents	Tallarook	5	328	93	1	6	1
	Tallarook*	5	196	98	—	2	0
	458	10	272	70	6	25	8
	458*	5	192	74	2	24	3
	Mt. Barker	6	219	83	4	13	5
	Mt. Barker*	5	200	87	1	12	1
	Dwalganup	8	223	68	5	27	7
	Dwalganup*	5	202	94	1	5	1
	N_1E	5	203	65	5	30	7
	Pink Flower	12	334	72	7	22	9
	Pink Flower*	4	160	88	5	7	5
F_1's	Tallarook × 458	5	314	23	25	51	52
	Tallarook × 458*	5	200	40	40	20	50
	Tallarook × Dwalganup*	5	199	72	17	11	19
	Tallarook × Pink Flower	4	116	66	7	27	10
	Tallarook × Pink Flower*	4	161	61	24	15	28
	Tallarook × Mt. Barker	3	88	89	3	8	3
	Tallarook × Mt. Barker*	4	158	91	2	7	2
	Mt. Barker × Dwalganup	6	234	62	15	23	20
	Mt. Barker × Dwalganup*	5	200	64	25	11	28
	Mt. Barker × Tallarook	5	135	92	—	8	0
	Mt. Barker × Tallarook*	5	200	93	3	4	3
	Yabba North × Yarloop*	4	117	73	1	26	1
F_2's	Tallarook × 458	19	391	47	17	35	27
	Tallarook × 458†	15	296	60	19	21	24
	Tallarook × Dwalganup	25	710	64	16	20	20
	Tallarook × Pink Flower	26	847	62	13	24	17
	Tallarook × N_1E	25	735	73	15	12	17
	Tallarook × Mt. Barker	25	849	92	3	5	3
	Mt. Barker × Pink Flower	26	993	46	22	32	32
	Mt. Barker × Dwalganup	21	569	57	23	20	29
	Mt. Barker × N_2E	27	585	64	19	17	23
	N_1E × Dwalganup	25	755	69	11	20	14

* Plants sampled by collection of 10 well-formed burrs. Remainder sampled by taking one runner.

† Excluding empty anthers.

The per cent. seed set estimated from the 10 burr samples would therefore be, and generally was, higher than the average for the whole runner. Nevertheless, the burr-sample method is probably to be preferred since an estimate, although slightly biased, is thus obtained of potential fertility, and real differences are less likely to be obscured by gross environmental variations. Results are presented in Table 2.

The correlations among per cent. plump seeds, per cent. empty flowers, per cent. seed abortion, and pollen sterility, derived from "runner sample" data of Table 2, are shown in Table 3 and Figures 2 and 3.

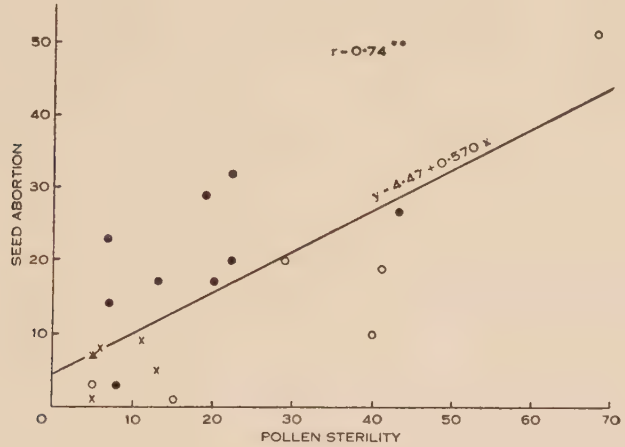


Fig. 3.—Relationship between pollen sterility (Table 1) and seed abortion (Table 2). × Parents. ○ F$_1$'s. ● F$_2$'s.

Sterility factors operating after fertilization are indicated by the presence of large numbers of shrivelled seeds in both F$_1$ and F$_2$ plants. Such embryo abortions are usually associated with endosperm failure (Brink and Cooper 1947). The aborted seeds are unlikely to represent all cases of embryo failure, for, as shown by Brock (1955), a large proportion of failure occurs shortly after fertilization. The numerous empty flowers in, for example, Tallarook × 458, may result from failure after fertilization rather than absence of fertilization. The correlation between seed set and pollen score (Fig. 2) may therefore reflect a common basis for pollen sterility and early embryo failure rather than pollen infertility *per se* especially as the number of pollen grains available is unlikely to limit fertilization.

The correlation between observed embryo abortion and pollen inviability (Fig. 3) may depend on the ratio of early to late abortions. Both pollen sterility and embryo abortion reflect the extent of incompatibility in these hybrids. In those hybrids in which sterility barriers are most severe only few balanced types would develop beyond early stages, and the ratio of "aborted" to "viable" seeds would probably fall, so that the correlation could become markedly curvilinear. The higher proportion of aborted seeds in the F$_2$ Mt. Barker × Pink Flower and Mt. Barker × Dwalganup relative to Tallarook × 458 and of F$_2$'s relative to F$_1$'s (Figs. 2 and 3) may be explained on this basis.

The existence of two ovules per ovary in this species (Aitken and Drake 1941) in addition to possible selection of balanced and viable female gametes (Renner effect) would further obscure relationships between male and female fertility. Normally only one ovule develops, and in so doing suppresses the other. If p is the potential proportion of viable zygotes, then potentially 0, 1, or 2 viable seeds per flower would be produced in $(1-p)^2$, $2p\,(1-p)$, and p^2 of cases respectively. The proportion of flowers actually developing seed would then be $p\,(2-p)$. Thus if p were 0·5, the actual seed production would be 0·75 (assuming all fertilized and viable zygotes developed).

Table 3

CORRELATIONS BETWEEN PERCENTAGE SEED SET AND PERCENTAGE POLLEN STERILITY

Correlation coefficients—data from Table 2. All values significant at 1% level

Character	Plump Seeds	Empty Flowers	Seed Abortion	Pollen Sterility
Plump seeds	—	−0·90	−0·90	−0·72
Empty flowers	−0·90	—	0·65	0·61
Seed abortion	−0·90	0·65	—	0·74
Pollen sterility	−0·72	0·61	0·74	—

Table 4

DEVELOPMENT OF BOTH OVULES IN SHRIVELLED OVARIES OF F_1 TALLAROOK × 458

20 burrs of F_1 and parents examined on November 17, 1954

Ovary Development				No. of Ovaries with 0, 1, and 2 Ovules		
Parents or F_1	Plump	Shrivelled	Empty Flowers	0	1	2
Tallarook	80	—	—	—	80	—
458	65			—	65	—
		1		—	1	—
			4	4	—	—
F_1	27			—	27	—
		27		—	17	10
			26	26	—	—

The presence of two ovules increases the possibility of selection operating both at fertilization, and during embryo development. This effect is demonstrated in the

F_1 of Tallarook × 458 (Table 4). Here all ovaries containing plump seeds had only one ovule developing. However, in 10 out of 27 ovaries containing shrivelled seeds two ovules were developing. Presumably these gametes were balanced and capable of being fertilized. However, sterility effects acting on both ovules after fertilization may have prevented full suppression of one by the other, so that both were represented as poorly-developed embryos.

The correlations between the pollen viability and the seed set among lines indicates that varieties, distinguishable by differences of subspecific rank in one character, tend to be distinguishable in other characters. But this finding does not indicate that the same factors operated to produce the different symptoms. This possibility can be tested by examining the segregation of different factors in the F_2 generation. Segregation for pollen sterility and seed setting in a number of F_2's is examined in Table 5.

TABLE 5

SEED SETTING IN F_2 PLANTS OF HIGH AND LOW POLLEN STERILITY

F_2	Pollen Sterility*	Plump Seeds (%)	Shrivelled Seeds (%)	Empty Flowers (%)	Seed Abortion (%)	No. of Flowers	No. of Plants
Tallarook × 458	Low	82	13	5	14	112	6
	High (49)	46	23	30	33	184	9
	Empty anthers	8	14	79	64	96	4
Tallarook × Dwalganup	Low	69	12	19	15	256	8
	High (29)	62	20	18	24	343	13
Tallarook × Pink Flower	Low	63	14	22	18	425	14
	High (24)	63	11	26	15	296	9
Mt. Barker × Dwalganup	Low	62	23	14	27	276	9
	High (32)	50	23	27	32	216	9
Mt. Barker × Pink Flower	Low	41	22	37	35	321	8
	High (23)	48	26	27	35	222	6

* Low, less than 10 %. High, greater than 10 %; averages in parenthesis.

In the F_2 of Tallarook × 458 there was a close relationship between the two types of abnormalities, which suggested a common cause, presumably genetic. However, in the other F_2's, this relationship was less evident or was absent, which suggested that the factors operating to produce pollen sterility and those influencing failure at or after fertilization were independent. Such contradictory results are difficult to reconcile, but may be considered in terms of interactions of possible processes, such as the "two-ovule effect", the Renner effect, linkage, segregation of univalents, and possible tapetum-pollen interactions.

The two-ovule effect and the Renner effect would diminish the correlation, but would not obscure it. If the production of normal gametes were determined by the balanced effects of many genes both on the same and on different chromosomes of a homozygous line, and if the balance were achieved in a different way in different lines for a number of different characters, hybridization, and subsequent recombination among genes, would destroy the necessary balance for all these characters. The incidence or severity of abnormalities would therefore tend to be correlated, being high in generally unbalanced individuals, low in generally balanced. Probably the most balanced individuals would be most like one of the parents. The greater the number of possible combinations which would be unbalanced for two characters, the higher the F_2 correlation would be. We would thus expect a tendency for higher correlations between the more complex characters and in the hybrids from the more divergent parents. Abnormalities determined by relatively simple mechanisms would occur more or less independently of others. This theory suggests that hybridization, by destroying the gene balance (both inter- and intra-chromosomal) produces correlated unfavourable effects by a mechanism similar to that suggested by Mather and Harrison (1949) to accompany intense selection. These authors demonstrated that in *Drosophila* extreme selection for one character in either direction could be accompanied by similar unfavourable responses in other characters. If a finely-adjusted genic balance exists in a species such as *Drosophila* and can be disturbed by selection, it is certainly likely to exist in normally self-fertilizing species, and to be disturbed by hybridization.

Individual plants with good seed set and high pollen sterility and vice versa occurred in each of the 5 F_2's studied. Therefore the two types of abnormality were determined by different genotypes on some occasions. Correlation between abnormalities may therefore express complexity of determination rather than common causation. This seems to be a reasonable explanation for variations among within-F_2 correlations.

(c) *Vegetative Abnormalities*

In two F_2 lines, dwarf and semi-dwarf individuals occurred (Table 6). No such plants were found in any of the parent lines, F_1's, or other F_2's. It is interesting that these two F_2's are among those which had the greatest pollen sterility.

Plants were classified as dwarfs, semi-dwarfs, and normals on October 2, and in late November plant diameters were measured (Table 7). The plant sizes in the relative classes generally confirm the ratings. However, subsequent to the time of rating, which was taken at the main period of flowering, some of the semi-dwarfs, and one of the dwarfs, made considerable vegetative growth. Further investigations are being made of the inheritance of the dwarf conditions in these crosses.

Table 7 also illustrates the vegetative vigour of the F_1's and the normal F_2's. The average end-of-season diameter of both the F_1's and F_2's of Tallarook × 458 exceeded that of either parent. F_1 plants of Tallarook × Clare grown in the glasshouse appeared correspondingly vigorous.

The relationship within F_2's between vegetative abnormalities and pollen viability is shown in Table 8. Within F_2's the correlation between these two characters was small and non-significant. However, the fact that the F_2's showing vegetative abnormalities were those showing greatest pollen sterility suggests that the former is a symptom of relatively extreme genetic divergence. The mechanism of dwarfing such as this is unknown, although dwarf and weak plants are frequently met in relatively wide crosses. If the genic balance and linkage theories described earlier are correct, the lack of correlation would indicate a relatively simple genetic determination of dwarfing. It is possible that these plants have small chromosomal deficiencies or duplications, but this possibility could not be investigated.

TABLE 6

PERCENTAGE OF ABNORMAL PLANTS IN THREE F_2'S AND PARENTS

Parents and F_2 Crosses	No. of Seedlings	Missing (%)	Dwarf (%)	Semi-dwarf (%)	Normal (%)
Tallarook	65	—	—	—	100
Clare	152	—	—	—	100
458	141	1	—	—	99
Tallarook × Clare	91	18	48	11	22
Tallarook × 458	122	6	21	—	73

TABLE 7

AVERAGES AND RANGES OF PLANT DIAMETERS OF PARENTS AND CROSSES WHICH SHOWED SEGREGATION OF DWARFS

Genotype	Type	No. of Plants	Average Diameter (in.)	Range (in.)	
				Smallest	Largest
458	Normal	45	37·5	26·0	48·0
Tallarook	Normal	43	40·5	28·5	52·5
Clare	Normal	62	37·9	26·5	50·5
Tallarook × 458 F_1	Normal	8	51·2	43·0	57·0
Tallarook × 458 F_2	Normal	43	41·9	32·5	54·5
Tallarook × 458 F_2	Dwarf	23	10·6	2·0	16·5
Tallarook × Clare F_2	Normal	20	42·2	26·5	51·5
Tallarook × Clare F_2	Semi-dwarf	8	26·1	19·5	35·5
Tallarook × Clare F_2	Dwarf	41	11·0	3·5	21·0 (one 30·5)

Data were not available to investigate the relationship between vegetative abnormalities and seed set. However, some dwarf plants in both crosses set seed readily and some vegetatively normal plants set relatively little seed. This supports the view that vegetative development and reproductive efficiency are virtually independent.

Other abnormalities were observed in F_2 plants of Tallarook × 458 grown by Dr. P. S. Nutman in studies on *Rhizobium*. Apart from dwarfs similar to those observed in the field, two dwarf plants produced leaflets which were very aberrant. The length was approximately twice the width, compared with the normal length: width ratio of about 0·9. One of these dwarfs had empty anthers and set no seed, the other had about 80 per cent. normal pollen and set some seed (Plate 1). In addition one plant of this cross was not only dwarf but did not flower during a 13 months life, despite exposure to a 20-hr day, under conditions which allowed all other plants in the glasshouse to flower.

TABLE 8

INDEPENDENCE OF VEGETATIVE ABNORMALITY AND POLLEN SCORE IN F_2

Abnormal Pollen Score (%)	Tallarook × 458 F_2		Tallarook × Clare F_2		
	Normal	Dwarf	Normal	Dwarf	Semi-dwarf
< 10	13	1	3	2	—
20–40	24	3	1	3	—
50–70	24	4	9	17	6
80–100	1	3	10	14	2
Empty anthers	11	3	—	—	—
Total	73	14	23	36	8

TABLE 9

CHIASMA FREQUENCY OF SOME PARENTS AND F_1'S

Parents and F_1 Crosses	No. of Cells	Mean Chiasmata per Cell	Chiasmata per Observed Bivalent
Tallarook*	19	15·9	2·02
458*	23	17·7	2·22
Clare	23	16·0	2·00
Wenigup	26	17·0	2·13
Burnerang × Wenigup	18	13·4	1·87
Tallarook × Clare	36	14·4	1·68
Tallarook × Wenigup	43	13·8	1·94
Tallarook × 458*	18	12·1	1·79

* Material collected in field, remainder from glasshouse.

(*d*) *Meiosis*

Meiotic behaviour was studied in four parent lines and the four F_1 hybrids which showed the greatest pollen sterility. Chiasma frequency was significantly reduced in each of the hybrids, indicating a degree of non-homology between the chromosomes or the genes of the parental lines (Table 9). The existence of chromosomal non-homology was confirmed by the metaphase associations (Table 10; Plate 2). The four parent lines each had a mean of approximately two chiasmata per bivalent and, except for occasional univalents in Tallarook, had regular pairing with eight bivalents. The hybrids had from 1·4 to 1·8 chiasmata per potential bivalent, 1·7 to 1·9 per observed bivalent, and pairing was far from regular. One of the hybrids, Tallarook × 458, had a high degree of failure of pairing; the other three showed evidence of structural changes indicating the presence of translocation heterozygotes.

Table 10

METAPHASE PAIRING IN SOME PARENTS AND F_1'S

Parents and F_1 Crosses	No. of Cells	Metaphase Pairing							
		1 IV 6 II	1 IV 5 II 2 I	1 III 6 II 1 I	1 III 5 II 3 I	8 II	7 II 2 I	6 II 4 I	5 II 6 I
Tallarook*	38		—	—	—	35	3	—	—
458*	117		—	—	—	117	—	—	—
Clare	23		—	—	—	23	—	—	—
Wenigup	26		—	—	—	26	—	—	—
Burnerang × Wenigup	18	6	—	5	1	4	2	—	—
Tallarook × Clare	36	6	—	9	2	12	5	2	—
Tallarook × Wenigup	43	17	3	9	2	10	1	1	—
Tallarook × 458*	51	—	—	—	—	36	13	1	1

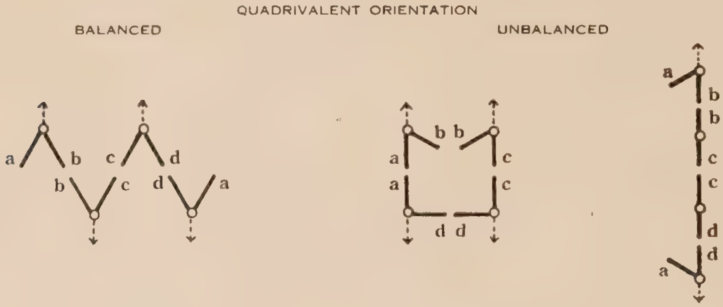
* Material collected from field, remainder from glasshouse.

This failure of pairing and structural hybridity leads to numerical and segregational unbalances in the products of meiosis, which could account for some of the observed pollen sterility.

Numerical unbalance results from random movement of univalents at meiosis. In the absence of polarized segregation 50 per cent. of the pollen grains will have a deficiency or an excess of chromosomes. Random segregation of univalents in both meiotic divisions was indicated by second anaphase (A II) counts of 10:8::7:7 and 8:7::9:8 in Tallarook × 458; 10:10::6:6 in Burnerang × Wenigup; and 7:7::9:9 in Tallarook × Wenigup. There was no evidence of misdivision of univalents which would add to the unbalance by the production of broken chromosomes.

Segregational unbalance resulting from duplications or deficiences of parts of chromosomes can follow certain types of anaphase separation of trivalent or quadrivalent associations. Unbalanced segregations of a trivalent and univalent are, in most cases, also numerically unbalanced. However, quadrivalent segregation usually results in numerical balance and the segregational balance depends upon the orientation of the quadrivalent (Plate 2; see also Sinnott, Dunn, and Dobzhansky 1950, p.255).

Figure 4 presents the types of quadrivalent orientations and the proportions of balanced and unbalanced types for each of the three hybrids. These data are insufficient to permit any differentiation between the hybrids on this basis, but, in general, about 80 per cent. of the quadrivalents result in segregational and numerical balance and consequently do not contribute to pollen sterility. The joint contributions of numerical and segregational unbalance of these F_1 hybrids (Table 11) accounts for less than 40 per cent. of the observed pollen sterility.



	F_1's	
5	BURNERANG X WENIGUP	1
6	TALLAROOK X CLARE	-
15	TALLAROOK X WENIGUP	5
26		6

Fig. 4.—Quadrivalent orientation and proportion which yield balanced and unbalanced products in the three translocation heterozygotes.

Summarizing we may say that there is evidence of non-homology, including translocations, of chromosomes of different varieties. The reduction in chiasma frequency per observed bivalent indicates the presence of rearrangements in several of the chromosomes, especially as the "Schultz-Redfield" effect (see White and Morley 1955) may operate to obscure such an effect, since rearrangements decreasing chiasma frequency in one chromosome or portion thereof tend to be accompanied by compensating increases in chiasma frequency in other portions of the chromosome complement. However, the structural rearrangements are insufficient to account for all, and probably less than half, of the observed pollen sterility.

IV. Discussion

This investigation has disclosed degrees of genetic incompatibilities in a rather limited set of crosses. These may be expressed in a variety of ways of which we have found reduced pollen fertility, reduced fertilization, embryo abortion, and vegetative breakdown. In general, these symptoms were correlated between crosses but within F_2 progenies most of the abnormalities appeared to be segregating independently.

Not all crosses showed symptoms of sterility barriers but when reproductive abnormalities occurred in a cross, all types of abnormality were usually present. Vegetative abnormalities only occurred in crosses showing severe reproductive abnormalities but pronounced reproductive failure was not invariably accompanied by vegetative breakdown.

Meiotic irregularities indicated structural chromosome changes between some parental varieties. Clare and Wenigup differed from Tallarook by at least one translocation. Similarly Burnerang and Wenigup differed by a translocation. The behaviour of the F_1 Tallarook ×458 indicated that some structural rearrangements had occurred but no translocations were observed.

TABLE 11

CONTRIBUTION OF NUMERICAL AND GENIC UNBALANCE TO POLLEN STERILITY (CALCULATED FROM TABLE 10 AND FIG. 4)

F_1	Numerical Unbalance (%)	Segregational Unbalance (%)	Total Unbalance (%)	Observed Pollen Sterility (%) (see Table 1)
Burnerang × Wenigup	22	7	29	85
Tallarook × Clare	25	3	28	80
Tallarook × Wenigup	19	9	28	80
Tallarook × 458	15	—	15	68

We have seen that these structural changes, as indicated by meiotic behaviour in the F_1, contribute to pollen sterility. However, their contribution in every case accounts for less than 40 per cent. of the observed pollen sterility (Table 11). Therefore most of the observed sterility is probably genic in origin or is determined by cryptic structural differences such as suggested by Stebbins and Vaarama (1954) in *Elymus* × *Sitanion* hybrids.

There are three kinds of evidence that support the view that the sterility is genic rather than chromosomal in origin.

Firstly, the variations in pollen fertility within and between anthers suggests a system of balance which is easily upset by small variations in the environmental conditions. A system so finely adjusted is likely to depend on balance between genes since the more gross structural effects would determine more generalized symptoms.

Secondly, predictions of the pollen fertility of crosses were not always fulfilled. For example, a study of the pollen fertility of the cross Tallarook × N_1E suggests that these varieties are fairly closely related, whereas Tallarook and Dwalganup are not (this is supported by Tallarook × Mt. Barker and Mt. Barker × Dwalganup crosses). From this one might predict that an N_1E and Dwalganup cross would have much pollen sterility. However, the pollen fertility of this cross was high. Such a situation is unlikely if the parents differ structurally.

Thirdly, the lack of correlation between abnormalities within F_2's suggests that finely balanced physiological systems were affected. If gross deficiencies or duplications were present one would expect more drastic symptoms.

While none of this evidence is conclusive it suggests that the observed sterility derives not only from chromosomal differences between varieties but also from disturbances to physiological equilibria determined by the balance of the individual genes.

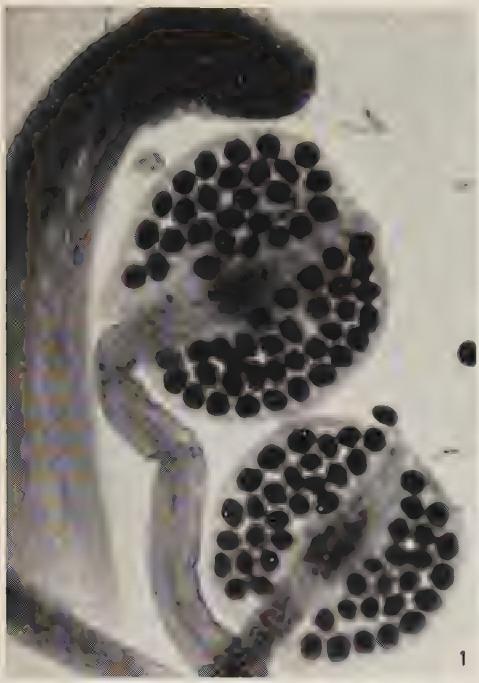
Unfortunately the geographical origin of the varieties in question is not known. Certain physiological studies in progress suggest that Tallarook, Mt. Barker, and Burnerang derive from areas which are climatically very different from those in which Wenigup, Clare, Yarloop, and the early-flowering varieties were originally evolved to their present forms. Crosses between Tallarook and the last three varieties mentioned certainly suggest genetic differences of a high order. Therefore the divergence is probably of long standing and their respective origins probably geographically remote. But the cross between Tallarook and N_1E, for example, i.e. between varieties which differ widely in maturity and in many morphological and physiological attributes, shows relatively little hybrid sterility. Therefore at least some types of physiological and morphological divergence need not be associated with a high degree of hybrid sterility. Obviously a clear picture of the role of geographic isolation in this case of subspeciation must await results of further work now being undertaken.

The accumulation of genic and structural differences of the order observed is very likely to have required large numbers of generations during which varieties were reproductively isolated. The period of 50 to 70 years in which the species has existed in Australia is probably quite inadequate. Therefore these observations support the contention of Aitken and Drake (1941) that the majority of the varieties existing in Australia were introduced as such. Nevertheless, the possibility should not be overlooked that occasional mutation or hybridization, followed by selection, may have resulted in some of the recognized strains.

The present range of *T. subterraneum* in Australia, and the agronomic value of the species, is largely determined by the combinations of characters existing in the available varieties. Although the species, and even single varieties, have been successful and extremely valuable in many habitats, there are strong possibilities that recombinations of characters will extend the range and increase productivity. The species is therefore of great potential value to the agronomist and plant breeder.

The importance of hybrid sterility in a programme of selection is difficult to evaluate. Extreme genetic divergence is probably an important barrier to hybridization. Nevertheless, crosses between widely divergent varieties (e.g. Tallarook and Clare) are possible, and these provide a wide range of recombinations. There is a possibility that in some desirable crosses the only balanced segregates will resemble one parent or the other, with no intermediate types. As seed production is likely to be an attribute of importance to the survival and use of the species there may be a strong case for rigid selection for seed yield during the early stages of a breeding

SUBSPECIATION IN TRIFOLIUM SUBTERRANEUM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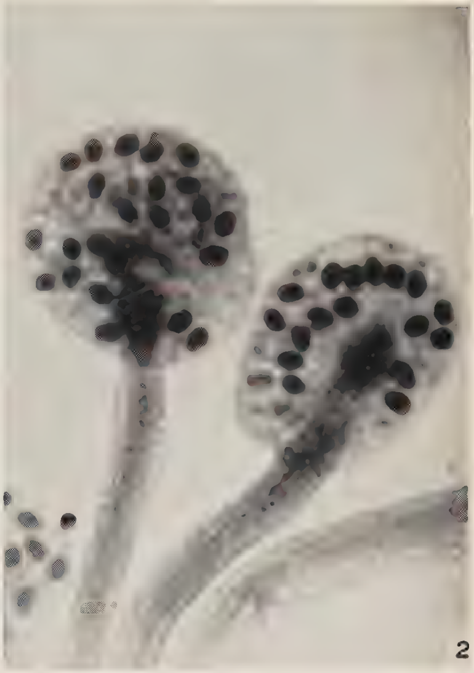
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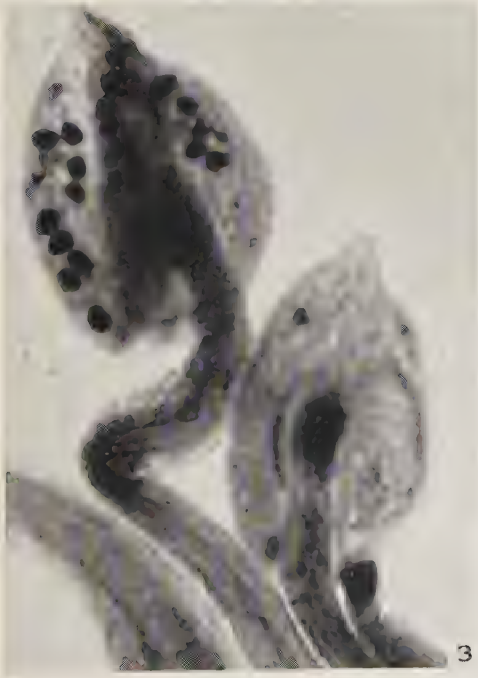
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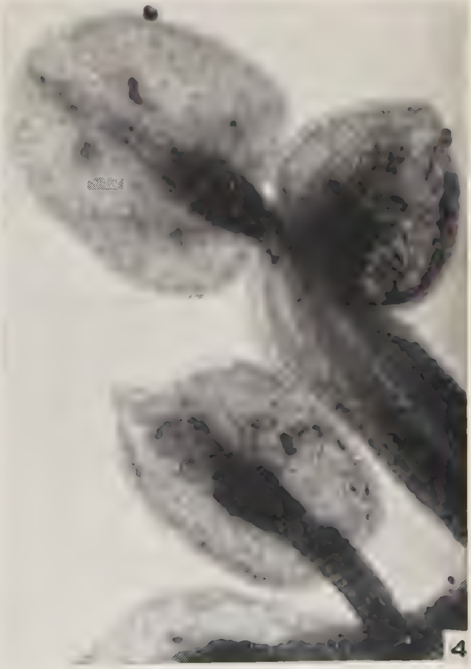
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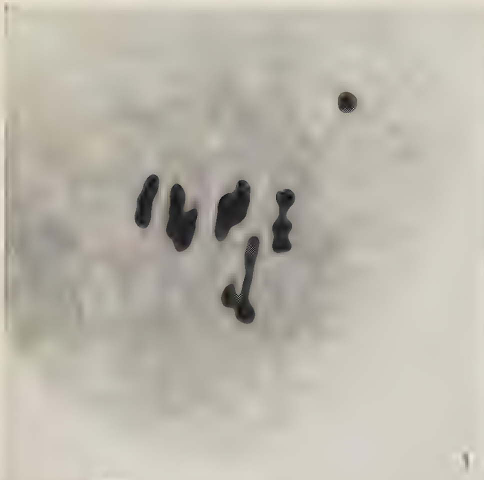

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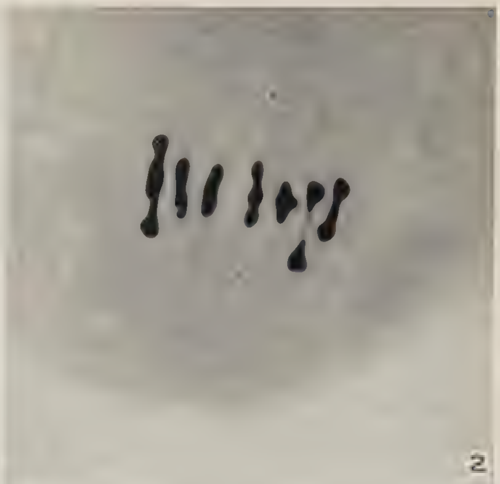

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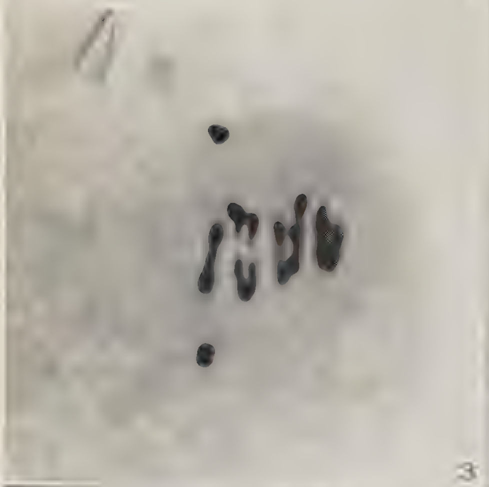

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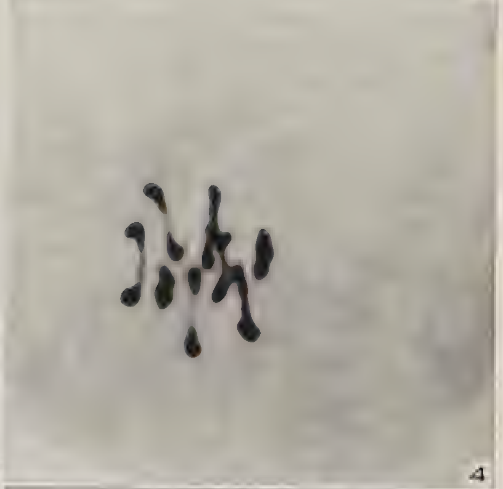

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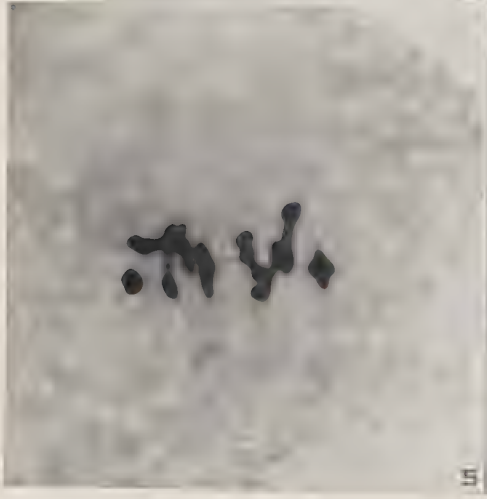

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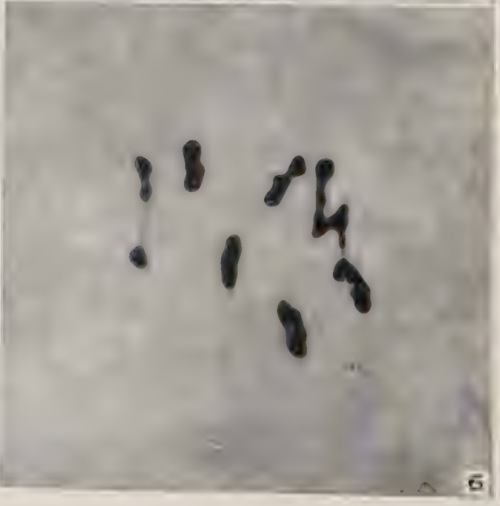

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programme. However, in plots exposed to natural selection there would be a fairly rapid elimination of plants of reduced fertility. Presumably such selection would lead to new genetically balanced and fertile strains.

V. ACKNOWLEDGMENTS

We are grateful to Dr. E. M. Hutton for provision of material. We wish to acknowledge the technical assistance of Mr. J. W. Peak who first drew our attention to the vegetative abnormalities in F_2's. Dr. P. S. Nutman kindly allowed us to use his experimental material.

VI. REFERENCES

AITKEN, Y., and DRAKE, F. R. (1941).—*Proc. Roy. Soc. Vict.* (N.S.) **53**: 342.
BAKER, H. G. (1951).—*Biol. Rev.* **26**: 302.
BRINK, R. A., and COOPER, D. C. (1947).—*Bot. Rev.* **13**: 423.
BROCK, R. D. (1953).—*Nature* **171**: 939.
BROCK, R. D. (1955).—*Heredity* **9**: 199.
CLAUSEN, J. (1951).—"Stages in the Evolution of Plant Species." (Ithaca: New York.)
DARLINGTON, C. D., and LA COUR, L. F. (1947).—"The Handling of Chromosomes." (Allen and Unwin Ltd.: London.)
DAVIES, J. G. (1951).—*J. Aust. Inst. Agric. Sci.* **17**: 54.
DONALD, C. M., and SMITH, C. A. N. (1937).—*J. Coun. Sci. Industr. Res. Aust.* **10**: 277.
FRANKEL, O. H. (1955).—*Caryologia* **6** (Suppt.): 600.
HUTTON, E. M., and PEAK, J. W. (1954).—*Aust. J. Agric. Res.* **5**: 271.
MATHER, K., and HARRISON, B. J. (1949).—*Heredity* **3**: 1, 131.
SINNOTT, E. W., DUNN, L. C., and DOBZHANSKY, T. (1950).—"Elements of Genetics." 3rd Ed. (McGraw-Hill Book Co. Inc.: New York.)
STEBBINS, G. L. (1950).—"Variation and Evolution in Plants." (Oxford Univ. Press.)
STEBBINS, G. L., and VAARAMA, A. (1954).—*Genetics* **39**: 378.
WHITE, M. J. D., and MORLEY, F. H. W. (1955).—*Genetics* **40**: 604.
YATES, J. J., and BRITTAN, N. H. L. (1952).—*Aust. J. Agric. Res.* **3**: 300.

EXPLANATION OF PLATES 1 AND 2

PLATE 1

Pollen grain development in some anthers of F_2 *T. subterraneum* hybrids. All × 65.

Fig. 1.—Normal anthers from Tallarook × Yarloop.
Fig. 2.—Reduced pollen fertility in Tallarook × Clare.
Fig. 3.—Extreme infertility in Tallarook × Clare. Note variation between anthers.
Fig. 4.—Empty anthers in Tallarook × 458. Note lack of differentiation. (cf. Figs. 2 and 3.)

PLATE 2

First metaphase configurations of some F_1 *T. subterraneum* hybrids. All × 1750.

Fig. 1.—Tallarook × Clare showing trivalent and univalent.
Fig. 2.—Tallarook × Wenigup showing quadrivalent.
Fig. 3.—Tallarook × Wenigup showing quadrivalent and two univalents. This type of quadrivalent orientation leads to segregational unbalance.
Fig. 4.—Burnerang × Wenigup showing quadrivalent.
Fig. 5.—Tallarook × Wenigup showing quadrivalent and two univalents.
Fig. 6.—Tallarook × Wenigup showing quadrivalent.

LINKAGE IN *LINUM USITATISSIMUM* AND IN *MELAMPSORA LINI* BETWEEN GENES CONTROLLING HOST-PATHOGEN REACTIONS

By G. M. E. Mayo*

[*Manuscript received May* 26, 1955]

Summary

The work of Flor, the most precise published on the genetics of disease resistance in higher plants, is shown to establish much less than is claimed. His allelic series are based on one case where linkage cannot be greater than 0·3 per cent. at 0·95 level of certainty, if the data are accepted as satisfactory: in fact, they contain several unsatisfactory features. For the rest of Flor's evidence, upper limits of linkages between genes within his allelic series of not less than approximately 20 per cent. are possible. More critical tests are needed, using progeny of heterozygotes backcrossed to a universally susceptible variety.

I. Introduction

Beginning with the discovery (Biffen 1907) of Mendelian inheritance of disease resistance and the recognition of physiological races in obligately parasitic fungi, for example in *Puccinia graminis* (Stakman and Piemeisel 1917), a great volume of work has been done on these subjects.

Originally, host varieties and progeny of their crosses were planted in field trials and their reactions to natural epidemics noted. Sometimes special nurseries were constructed to encourage these natural epidemics. Then followed efforts to separate mixed inocula into pure strains (often referred to as "races"), identified by their reaction against a set of host strains which came to be adopted as the "differentials" in such tests. More recently, there has been a trend towards listing the host genes conferring resistance rather than, less specifically, host strains, each of which may contain one to several such genes.

In flax (*Linum usitatissimum*), the last phase has been elaborated especially by Flor (1942, 1946, 1947). Following Henry's (1930) finding that immunity† was conditioned by a single dominant factor in the varieties Bombay and Ottawa 770B and by either of two dominant factors in the Argentine selections he tested, Myers (1937) postulated two allelic series of host factors conferring resistance or immunity.

Flor (1941, 1946, 1947, 1954) modified and extended Myer's original hypothesis to three series of multiple alleles or groups of very closely linked genes in the host. He also claimed to have found linkage between host-range genes, in some cases very close.

Although there are data available from Australian sources (e.g. Finlay 1953; Pugsley and Carter 1953; Carter 1954; Mayo (unpublished data)), none are so extensive, including both host and pathogen, nor precise as those published by Flor. For this reason these data have been used in the following analysis. This analysis attempts to show that there is a widespread misunderstanding in plant breeding literature about the amount of information available per individual from F_2 linkage data.

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* Department of Genetics, University of Adelaide.

† In this paper, the terms "immune", "resistant", "susceptible", "avirulent", and "virulent" are used in the sense of Flor (1942).

II. FLOR'S CONCLUSIONS

Flor (1935, 1940, 1941, 1942, 1946, 1947), beginning in 1931, collected a wide range of host and pathogen varieties and used them to analyse the genetics of host resistance and of pathogen virulence. From this work he concluded that:

(i) The reaction due to each host gene for immunity, resistance, or susceptibility is sufficiently clear cut to suggest that secondary effects are absent.

(ii) Specificity of reaction, previously envisaged as between host variety and pathogen "race", is more precisely related to genes in the host controlling resistance and genes in the pathogen controlling avirulence.

(iii) This being so, there appears to be no theoretical limit to the accumulation in *Melampsora lini* of genes for virulence and the evolution of a strain capable of attacking all varieties of flax.

(iv) On the other hand, pathogen strains arising through hybridization will not attack host strains previously resistant to both parental strains of the pathogen.

(v) Most of the host genes controlling rust reactions lie in three allelic or closely linked series. In fact, Flor adopted a nomenclature appropriate to allelic series: *L* and *M* were named, following Myers though with some modifications, and *N* was added. Up to 1947, Flor claimed to have identified L, L^1, ...L^7; M, M^1, ... M^6, and N, N^1 . . . N^5, conferring immunity or resistance and dominant to l, m, and n respectively, conferring susceptibility. These are recognized by the reactions shown by appropriate test varieties of *M. lini* (Table 1).

(vi) In all but one (see Section V) of at least 18 genes identified by Flor (1946) as affecting rust reaction in the pathogen, avirulence is dominant to virulence. These 18 genes are claimed to be linked in 12 independently inherited groups.

(vii) Finally, Flor (1947) claims that "there is a parallelism in linkage between certain factors for rust reaction in the host and those for pathogenicity in the fungus." For example, virulence of F_2 pathogen cultures segregated identically when tested on Akmolinsk (N^1N^1), Abyssinian (N^2N^2), and Leona (N^3N^3) host varieties having allelic factors. "However, pathogenicity to these three varieties having factors in the *NN* series was also linked with that to Buda ($L^1L^1M^2M^2$), indicating that linkage between pathogenic factors transcends the linkage groups conditioning resistance to the host."

III. NATURE OF FLOR'S EVIDENCE

In collecting the data on which he based these conclusions, Flor enormously increased the information obtained per plant grown, by carrying out tests of host-pathogen reaction successively on the same plant, inoculating with less virulent pathogen stocks first. Conversely, he tested progeny of pathogen crosses against an array of host differentials. A simple hypothetical example* is given in Table 2.

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* Flor's (1947) use of a relatively large number of pathogen strains between different segregations prevents the use of actual data in a simple example.

TABLE 1

DIFFERENTIAL REACTIONS OF HOST GENES RECOGNIZED BY FLOR

Compiled from Flor (1945, 1947) assuming the reactions are due only to the genes which have been identified. I = Immune; R = resistant; S = susceptible

Gene	Race																											
	1	2	3	4	5	6	7	8	9	16	19	20	21	22	24	41	47	52	55	60	68	73	75	79	81	85	105	111
L	I	I	I	I	I	I	I	I	I	I	I	I	I	S	I	I	S	I	S	I	S	S	I	I	I	I	I	I
L^1			S	S	R		$*^1$		S−	S	S	I	S	S		$*^2$	S	S	S	S	S	S	S	S	S	S	S	R
L^2	I	I	I	I	I	I	S	I	S	S	I	I	S	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
L^3	R−	R−	R−	R−	R−	R−	R−	R−	R−	R−	S	S	R−	S	R−	R−	R−	R−	R−	R−	R−	R−	R−	S	S	S	S	S
L^4	R	R	SR	R	R	R	R	SR	R	R	S	S	SR	S	R	R	R	R	R	R	R	R	R	S	S	S	S	S
L^5	R	S			$*^3$	S	$*^1$			R	S	S	S	S	S	$*^3$	S	S		S	S	S	S	S	S	S	S	S
M	I	I	I	I	I	I	I	I	I	I	S	S	I	S	I	I	S	I	I	S	I	S	I	I	I	S	I	S
M^1	S	S	S−	S−	$*^3$	S	R	S−		S	S	S	S	S	S	$*^3$	R	R	R	S	S	S	S	S	S	S	S	S
M^2			SR	S	R		$*^1$?		S−	S	S		S	S		$*^2$	S	S	S	S	S	S	S	S	S	S	S	R
M^3						$*^4$	I			I	S	S	$*^1$	S	I	I		$*^1$		I	$*^4$					S	S	
N	I	I	I	I	I	I	I	I	I	I	I	I	I	I	S	S	I	I	S	I	I	S	I	I	I	I	I	I
N^1	R	R	S	R	S	R	S	S	R	S	S	S	S	S	R	R	R	S	S	R	R	R	R	R	R	R	S	S
N^2	I	I	R	I	R	I	R	S	I	$*^2$	S	S	S	S	I	I	$*^1$	S	S	I	$*^1$	I	I	I	I	I	S	R
N^3	R	R	R	R	R	R	R	R	R	R	S	S	R	S	R	R	R	S	S	R	R	R	R	R	R	R	S	R
N^4	I	I	I	I	I	I	I	I	I	I	S	S	I	S	I	I	R	S	I	I	I	I	S	I	I	I	I	I

$*^1$ = I to R; $*^2$ = S− to S; $*^3$ = R to S; $*^4$ = I to S−.

In this table, the fitted segregation ratios are given of only three host strains (*W*, *X*, and *Y*), and the progeny of their crosses tested against two pathogen strains (*a* and *b*) and the F_2 progeny of a cross between these strains. The same reaction, that of host-pathogen, is used and the result looked at differently according to whether the viewpoint of the host (immune, resistant, or susceptible) or the parasite (avirulent or virulent) is being taken. The host represents the medium on which the fungus grows, and it is of interest to know what in each leads to a negative reaction (i.e. no, or very little growth). The analysis of differences between different hosts and between different parasites proceeds by keeping the tester parasites and tester hosts, respectively, constant. This is the assumption underlying the use of a set of host "differentials" and a set of parasite "testers".

Flor's analysis of these data would proceed as follows: The progeny of $X \times Y$ show consistency with the hypothesis that one gene is segregating with respect to *b* and none for *a*; in $X \times W$, two genes are segregating independently, one with respect to *a* and one for *b*, while in $Y \times W$, two genes are segregating, one with respect to *a* and both for *b*. Failure to recover any susceptible F_2 plants from the two immune parents *X* and *Y* with respect to *a* (cf. $Y \times W$ with respect to *b*) is accepted by Flor as a test indicating the presence of allelic or very closely linked genes in each host.

The F_2 progeny derived from the pathogen crosses $a \times b$ show segregation for one gene when tested against hosts *W* and *X* and no segregation when tested against *Y*.

Considering the host and pathogen data together, it is concluded that avirulence is dominant to virulence and immunity or resistance dominant to susceptibility, and that there is no interaction between genes. Using italic capitals to denote dominant host genes and Greek capital letters to denote dominant pathogen genes, Flor's analysis of host and pathogen genotypes may be tabulated (Table 2, Part *II*).

From $X \times W$, *X* has a gene (*B*) for immunity to *a* and *W* a gene (*A*) for immunity to *b*: hence *X* is *aaBB* and *W* is *AAbb*. From $Y \times W$, *Y* has a gene (*C*) for immunity to *a* and *b*, and *W* has a gene (*D*) for immunity to *b*: hence *Y* is *CCdd* and *W* is *ccDD*. Since *A* and *D* both occur in *W* and confer the same properties of host immunity to the pathogen strain *b* and susceptibility to *a*, Flor considers that $A = D$, that they are identical, and are notated as *A* in Table 2, Part *II*. Again, F_2 progeny of $X \times Y$ fail to show any segregation with respect to *a* and, presumably, *X* and *Y* each contain at least one gene for immunity to that strain: furthermore, Flor considers that these genes must be identical, allelic, or linked (Flor claims very closely linked) since there is no segregation. The crosses of *X* and *Y* to *W* have obviously shown *X* to possess *B* and *Y* to possess *C*, each conferring immunity to *a*: Flor considers it simplest to assume that there are two genes involved in preventing segregation in the F_2 of $X \times Y$ inoculated with *a*. In doing so, since *B* and *C* confer different properties with respect to *b*, Flor reduces the alternative explanation of no segregation to allelism or close linkage between *B* and *C*. Flor argues that since recombination is practically unknown we have the equivalent for practical purposes of allelism and he adopts an appropriate notation. Thus, in Table 2, Part *II*, *C* is shown as B^1.

The analysis to identify genes in the parasite is similar. In this case, Flor marks the genes with superscripts to relate them to the appropriate host tester (Table 2, Part *II*). It was from this stage of his analysis that Flor drew the second con-

TABLE 2

FLOR'S (1946, 1947) INTERPRETATION OF HOST-PATHOGEN REACTIONS BETWEEN L. USITATISSIMUM AND M. LINI

Host phenotype: I = immune; R = resistant; S = susceptible. Pathogen phenotype: A = avirulent; V = virulent. I and R equivalent to A. S equivalent to V

I. Host-Pathogen Reactions

(i) Host Reaction to Two Strains of *M. lini*

Host Cross 1×2	Pathogen Tester Strain	Host Reaction			
		Host 1	Host 2	F_1	F_2
$X \times Y$	a	I	I	I	I I
	b	S	I	I	I S
					3 : 1*
$X \times W$	a	I	S	I	I I S S
	b	S	I	I	I S I S
					9 : 3 : 3 : 1*
$Y \times W$	a	I	S	I	I S S
	b	I	I	I	I I S
					12 : 3 : 1*

(ii) Pathogen Reaction to These Strains of *L. usitatissimum*

Pathogen Cross	Host Tester	Pathogen Reaction			
		a	b	F_1	F_2
$a \times b$	W	V	A	A	3A : 1V
	X	A	V	A	3A : 1V
	Y	A	A	A	all A : 0V

II. Genotype Deduced from Host-Pathogen Reactions

(i) Host

Host Strain	Inferred Genotype	Reaction of Dominant Gene with Pathogen Strains a and b		
		Gene	a	b
W	$AAbb$	A	S	I
X	$aaBB$	B	I	S
Y	aaB^1B^1	B^1	I	I

(ii) Pathogen

Pathogen Strain	Inferred Genotype	Reaction of Dominant Gene with Host Strains W, X, and Y		
		Gene	W	X	Y
a	$\Gamma^X\Gamma^X\Delta^Y\Delta^Y\pi\pi$	Π^W	A	V	V
b	$\gamma\gamma\Delta^Y\Delta^Y\Pi^W\Pi^W$	Γ^X	V	A	V
		Δ^Y	V	V	A

* Fitted ratios.

clusion. In this case, from Table 2, Part *II*, pathogen gene Γ^X appears to correspond to host gene *B*, Δ^Y to B^1, and Π^W to *A*.

This was the nature of the evidence on which Flor based all his conclusions. This elegant method has been developed and used systematically by Flor over the last twenty years so that he has been able to demonstrate a more comprehensive and complete picture of the inheritance of resistance to rust in flax than any previously achieved. However, it will be shown that the evidence leaves room for alternative explanations.

IV. Evidence for Allelism between Host Genes

Flor's fifth conclusion (loc. cit.) was that there are three series of genes, either strictly allelic or very closely linked. Within a series, the several genes for resistance are distinguishable by the different varieties of pathogen to which they confer resistance (Table 1). Such a range of difference within a series of alleles suggests the necessity to examine the strength of the evidence for allelism or very tight linkage. In Table 3 are set out the F_2 expectations for allelism, independence, and linkage of two dominant genes, one in each of two varieties, by which each differs from a third variety. Thus, in example 1, host varieties *B* and *C* each differ from *A* by a dominant gene for immunity, and different segregations are expected according to whether these genes are allelic or not, and according to whether they are linked or not. However, with an F_2 family of limited size, discrimination between these possibilities becomes uncertain. It is especially difficult to separate linkage from allelism in those cases where, as in the examples of Table 3, the test is carried out with the genes in the repulsion phase, since the frequency of the detectable class of recombinant is only a quarter of the squared value of the recombination fraction.

With close linkages, plant breeders would encounter difficulties in combining two resistant genes in one variety. Thus Flor (1947) claims that "it appears unlikely that more than 1 pair of the *LL* or *MM* factors can be incorporated into a line."

Examination of Flor's data (1941, 1947) shows that:

(i) No test deals with progeny derived from a cross where the phenotype of one parent is universally susceptible as in the case of *A* in Table 3. They involve only crosses between parents both of which are resistant (or immune) to at least one of the pathogen strains, as in the cases of *B*, *C*, *D* and *E* in Table 3. Once the idea of allelism became accepted, it is easy to understand how interest would have centred on testing whether two genes for resistance in different varieties were allelic or not, rather than whether a resistant variety differed from a susceptible one by one gene or by two linked genes. Such tests of allelism, involving F_2 segregations from a repulsion phase heterozygote, are very inefficient.

(ii) These tests involve only F_2 segregations, except for one case (Flor 1941) in which two sets of F_3 families were also tested. The test for allelism is based on an expectation of all: 0, 2: 1: 1: 0, or 3: 1: 0 as distinct from 15: 1, 9: 3: 3: 1, or 12: 3: 1 respectively, where free recombination occurs. Where linkage occurs, with a recombination fraction of p, the expectation for the detectable recombinant segregates that would fail to appear if instead the genes under test are allelic, would be $p^2/4$ in

TABLE 3

F_2 SEGREGATION DIFFERENTIATING INDEPENDENCE, ALLELISM, AND LINKAGE BETWEEN HOST GENES TESTED AGAINST PATHOGENS a AND b

I = Immune; R = resistant; S = susceptible; p = recombination fraction

Example	Host	Host-Pathogen Reaction		Cross	Expected Segregation Based on:												Host	Genotype Based on:		Gene	Host-Pathogen Reaction	
					Independence				Allelism				Linkage					Non-allelism	Allelism			
		a	b		a: I b: I	I S	S I	S S	I I	I S	S I	S S	I I	I S	S I	S S					a	b
1	A	S	S	$A \times B$			$\frac{3}{4}$	$\frac{1}{4}$			$\frac{3}{4}$	$\frac{1}{4}$			$\frac{3}{4}$	$\frac{1}{4}$	A	$llxx$	l l	L	S	I
	B	S	I	$A \times C$			$\frac{3}{4}$	$\frac{1}{4}$			$\frac{3}{4}$	$\frac{1}{4}$			$\frac{3}{4}$	$\frac{1}{4}$	B	$llXX$	L^x L^x	L^x	S	I
	C	S	I	$B \times C$			$\frac{15}{16}$	$\frac{1}{16}$			1	0			$\frac{1-p^2}{4}$	$\frac{p^2}{4}$	C	$LLxx$	L L	X	S	I
2	A	S	S	$A \times B$			$\frac{3}{4}$	$\frac{1}{4}$			$\frac{3}{4}$	$\frac{1}{4}$			$\frac{3}{4}$	$\frac{1}{4}$	A	$llxx$	l l	L	S	I
	B	S	I	$A \times D$		$\frac{3}{4}$		$\frac{1}{4}$		$\frac{3}{4}$		$\frac{1}{4}$		$\frac{3}{4}$		$\frac{1}{4}$	B	$LLxx$	L L	L^x	I	S
	D	I	S	$B \times D$	$\frac{9}{16}$	$\frac{3}{16}$	$\frac{3}{16}$	$\frac{1}{16}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{4}$	0	$\frac{2+p^2}{4}$	$\frac{1-p^2}{4}$	$\frac{1-p^2}{4}$	$\frac{p^2}{4}$	D	$llXX$	L^x L^x	X	I	S
3	A	S	S	$A \times B$			$\frac{3}{4}$	$\frac{1}{4}$			$\frac{3}{4}$	$\frac{1}{4}$			$\frac{3}{4}$	$\frac{1}{4}$	A	$llxx$	l l	L	I	I
	B	S	I	$A \times E$	$\frac{3}{4}$			$\frac{1}{4}$	$\frac{3}{4}$			$\frac{1}{4}$	$\frac{3}{4}$			$\frac{1}{4}$	B	$llXX$	L L	L^x	S	I
	E	I	I	$B \times E$	$\frac{12}{16}$		$\frac{3}{16}$	$\frac{1}{16}$	$\frac{3}{4}$		$\frac{1}{4}$	0	$\frac{3}{4}$		$\frac{1-p^2}{4}$	$\frac{p^2}{4}$	E	$LLxx$	L^x L^x	X	S	I

each case (Table 3). For a given family size, the limits of p at 0·95 level of certainty may be calculated. Where, as occurred in all but two cases involving Flor's N series, the observed value of $p^2/4$ is zero, the lower limit of p is also zero: but the upper limit is dependent on family size and the way in which it varies is plotted in Figure 1. It will be seen that for most of the F_2 families analysed, the upper limit of recombination excluded at 95 per cent. certainty does not fall below a value of about 20 per cent. The evidence, therefore, does not exclude even fairly loose linkage.

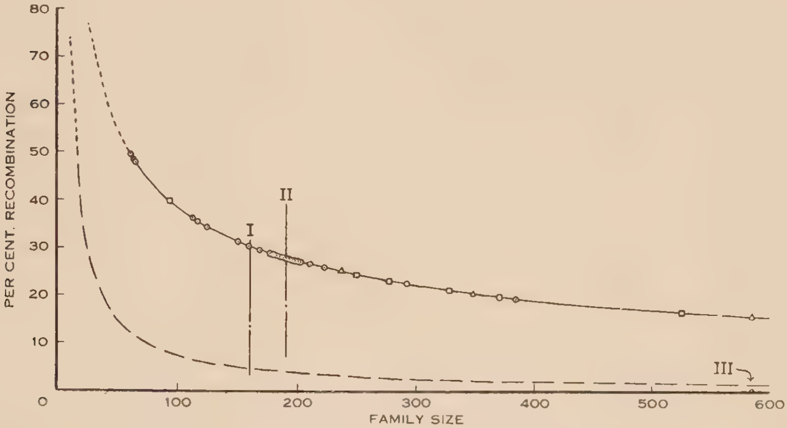

Fig. 1.—Upper limit of recombination fraction at 0·95 certainty for different size of F_2 and backcross families from heterozygotes in repulsion phase. —— F_2 values. ------ Backcross values. Actual data: △ (Flor 1941), ○ (Flor 1947), □ (Mayo, unpublished). Estimates and limits of recombination for Bombay × Akmolinsk F_2, and for Leona × Bombay F_2 (data from Flor 1947) are shown as I and II ($p=13{\cdot}3$ and $17{\cdot}7$ per cent. respectively). Upper limit from Buda × J.W.S. F_3 (data from Flor 1941) is shown at III.

In two cases, rare recombinants occurred (Flor 1947), so that estimates of actual values for the recombination fraction, as well as their upper and lower limits, may be calculated. These are represented at I and II in Figure 1 for convenient comparison with the rest of the data yielding information on allelism, though their calculation is quite separate.

Flor supposed that even if the presumed recombinants are disregarded, the irregular ratios shown by significant deviations from 2: 1: 1: 0 or equivalent ratios are themselves evidence of linkage and therefore of the segregation of two genes which a 2: 1: 1: 0 ratio excludes. In fact, disturbances due to joint segregation of two allelic genes are not independent of aberrant segregations of the individual genes. However, testing the same data against an expectation that the two genes segregate independently giving a joint segregation of 9: 3: 3: 1 provides functions which are orthogonal and which permit an analysis of χ^2 into its components. Table 4 analyses those segregations of N genes (Flor 1947), including the two with recombinants. Evidently the major consistent disturbance is due to linkage between N and N^x. However, a further significant disturbance in two of the segregations is due to a locus showing poor single gene ratios. This suggests difficulties of classification absent from Flor's (1947) other published F_2 data which show no disturbance due to linkage.

Table 4

PRINCIPAL EVIDENCE OF DISTURBED SEGREGATIONS IN PROGENY OF CROSSES BETWEEN HOST VARIETIES CARRYING GENES OF FLOR'S N SERIES

Values of χ^2 at 5 per cent. probability = 3·841, 5·991$_{(2)}$, 7·815$_{(3)}$. Assumptions: Gene segregating derived from Bombay and Punjab 5 = N. N^x = N^1 in cross 1, N^3 in cross 2, and N^4 in cross 3.

I = Immune, S = susceptible, I/R = immune or resistant

(*a*) Data (extracted from Flor 1947)

Cross: 1 2 3	Host Reaction				Total F_2 Plants	χ^2 (based on 9:3:3:1)
Race: 5 52 19 Race: 41 73 41	I I/R	I S	S I/R	S S		
Cross	Gene Segregating					
	N and N^x	N	N^x	—		
1. Bombay × Akmolinsk F_2	72	44	43	1	160	23·867$_{(3)}$
2. Bombay × Leona F_2	85	39	64	2	190	35·803$_{(3)}$
3. Punjab 5 × Tammes Pale Blue F_2	114	30	48	0	192	17·333$_{(3)}$
Total (1+2+3)	271	113	155	3	542	61·211$_{(3)}$

(*b*) Components of χ^2

Cross	Contribution to χ^2 of:		
	N:n	N^x:n	Linkage
1	0·533	0·833	22·500
2	9·606	1·186	24·815
3	0·000	9·000	8·333
Total	10·139$_{(3)}$	11·019$_{(3)}$	55·648$_{(3)}$
Deviation	4·982	3·742	52·492
Heterogeneity	5·157$_{(2)}$	7·277$_{(2)}$	4·168$_{(2)}$

(iii) Flor's most stringent test to establish allelism arises from his work (1941) on two sets of F_2 and F_3 progeny of reciprocal crosses between Buda and J.W.S. tested against races 4 and 7 in the first set, and 3, 4, and 7 in the second set. Both F_2 families were large, including respectively 348 and 237 plants giving 348 and 236 F_3 families that were tested, the latter totalling 12,641 plants.

However, the evidence is marred by a number of unsatisfactory features. In addition to the segregation of the two genes claimed by Flor to be allelic and belonging to his L series, an additional dominant gene (R) derived from Buda and conferring resistance to race 7 and "semi-resistance" to race 3 is segregating. The classes of segregants that indicate the presence of this additional gene contribute no information determining allelism between the other two genes.

The "allelic" genes, L^1 derived from Buda and L^2 from J.W.S., each confer identical host reactions with respect to races 3 and 4: therefore, segregants with respect to race 4 only are tabulated in Table 5. For race 7, L^1 confers host immunity whilst L^2 confers susceptibility. These susceptible reactions become modified to resistant when R is present in the host. The segregation of L^1 and L^2, therefore, is fully described if segregants susceptible and resistant to race 7 are pooled in the same class as in Table 5.

Though L^1 and L^2 are postulated to be allelic, tests in both F_2 populations (Table 5) are consistent in the disagreement of the observed joint segregations with the expected 2: 1: 1: 0 ratio. Flor ascribes this disagreement to inconsistent expressivity of the allele derived from J.W.S. In any event, it is not material to the fact that the only evidence consistent with Flor's theory of allelism in these F_2 data is that no plant showed susceptibility to both races. The upper limit of recombination that could occur in such data, at a level of certainty of 0·95, when genes whose loci are linked are present, is shown in Figure 1 to be not less than 16 per cent. even when assuming that the two F_2 segregations are homogeneous.

In the F_3 data, 34 families in the first test and 17 in the second all derived from F_2 parents previously classified as immune to both races show no segregation and are all immune to race 4 and resistant or susceptible to race 7. Since this result is impossible to interpret by any simple hypothesis of major gene segregation, either for allelic, independent, or closely linked segregation, Flor accepted this as evidence of misclassification of the F_2 parental plants. He therefore reclassified them (Table 5) and showed that his F_3 families were in good agreement with a hypothesis of allelism. In fact, the fit of the F_2 plants now becomes remarkably good.

Using these reclassified data, the upper limit of p, the recombination fraction, at a 0·95 level of certainty calculated from the pooled F_3 data, taken to be homogeneous for both tests, is reduced to just over 0·3 per cent (III in Fig. 1). This upper limit was determined by equating the sum of the recombinant segregates detectable by F_3 segregation, $(4p-3p^2)/2$, to 0 and determining the upper limit of p consistent with observing no recombinant from an F_2 family size of 584 at 0·95 level of certainty.

The fact that all the detected misclassifications with respect to race 7 occurred only with F_2 plants immune to race 4 and not with those susceptible has been accepted by Flor as further evidence of allelism. However, it may have been due to the relatively low expectation of the genotype, susceptible thus to misclassification, amongst data in the collection of which Flor himself admits having difficulties.

Myers' (1937) original evidence of allelism is based exclusively on F_3 data that are even less satisfactory than Flor's. Only 11 of the 37 crosses tested by Myers could contribute to his hypothesis and in these the data are often unsatisfactory. Frequently it is difficult to relate Myers' elaborate numerical classification of host-pathogen reaction to his parallel descriptive classification. In five of the 11 crosses, there is at least some evidence of segregation admitted by Myers to throw doubt on the evidence

TABLE 5

REACTION TO PATHOGEN RACES 4 AND 7 OF F_2 PLANTS AND F_3 FAMILIES DERIVED FROM BUDA × J.W.S.

Analysis of data from Flor (1941) on the assumption of a 2:1:1:0 segregation ratio (see Section IV (ii) and (iii)). I = Immune, S = susceptible, R/S = resistant or susceptible

Host	Race	Reaction to Pathogen					Total	χ^2	P
	4 7	I I		I R/S	S I	I S			
Parents F_2 Plants				J.W.S.	Buda				
Family 1		209		51	88	0	348	$21{\cdot}948_{(2)}$	$< 0{\cdot}001$
2		140		33	64	0	237	$15{\cdot}511_{(2)}$	0·01-0·001
								$37{\cdot}860_{(4)}$	
Total (1+2)		349		84	152	0	585	$37{\cdot}636_{(2)}$	$< 0{\cdot}001$
								$0{\cdot}224_{(2)}$*	0·98-0·95
F_3 Families derived from F_2:	4 7	I I S S I I 1 : 2 : 1	I R/S	I R/S	S I	S S			
Family 1		175	34	51	88	0			
2		123	17	32	64	0			
F_2 Plants classified by means of F_3:	4 7	I I	I R/S		S I	S S			
Family 1		175	85		88	0	348	$0{\cdot}063_{(2)}$	>0·99
2		123	49		64	0	236	$2{\cdot}326_{(2)}$	0·7-0·5
								$2{\cdot}390_{(4)}$	
Total (1+2)		298	134		152	0	584	$1{\cdot}356_{(2)}$	0·8-0·7
								$1{\cdot}034_{(2)}$*	0·8-0·7

* Heterogeneity χ^2.

for allelism. Furthermore, Myers used a field collection of the pathogen and a stock, "form 4", which he claimed behaved identically for purposes of analysis, but this is not borne out by his own results.

A reanalysis will not be presented here, since even if the data are accepted without reserve, they contribute very little to establish allelism. The F_3 families cannot be quoted in relation to their F_2 parents so that the evidence for allelism is only as strong as the largest non-segregating F_3 family: in no quoted case is this above 36 plants or the equivalent to an upper limit of free recombination.

Thus, it is clear that Flor's series of five to seven alleles at each of three independent loci is founded most strongly in one case, in which linkage, as an alternative to allelism, is restricted to a recombination of less than 0·3 per cent. derived from experiments where difficulties in classification were encountered. Collaterally it is founded also on a relatively large number of cases when linkage could be present up to a limit of not less than 16 per cent. That is, from data showing one doubtful case of allelism, an extensive range of three allelic series has been postulated.

V. Evidence for Linkage Between Pathogen Genes

In the pathogen, Flor (1946) assumes 12 linkage groups between which the 18 identified genes for avirulence are distributed. This assumption is based on two sorts of evidence:

(i) F_2 cultures were shown to segregate, when tested against two or more host varieties, so that class numbers for each variety corresponded, pathogen culture by pathogen culture. Included in this category are A_{ak}, A_{ab}, and A_{le}, and A_p and A_k (Table 6). Flor distinguishes between these genes that are "inherited on a unit basis" (Flor 1954) in three ways:

(1) The host-pathogen reaction differs between host testers. For example, the presence of A_{le} is characterized by a resistant reaction whilst that of A_{ab} is immune or resistant (Table 6).

(2) The unit basis of inheritance may break down. In the only case in his data, A_{ak}, Flor (1946, p. 349) claims that "it is necessary to assume a factor for pathogenicity on Akmolinsk distinct from those on Abyssinian and Leona in order to explain the pathogenicity of hybrid A". This breakdown in correspondence between segregating classes is complete (i.e. Akmolinsk was susceptible to all F_2 cultures of hybrid A of race 6 × race 22) and does not show recombination between incompletely linked genes.

(3) Though the genes in the pathogen segregate over two or more hosts as a unit, if it is accepted that the host genes, in each case, differ, then it is probable that the pathogen genes do also. In the case of A_{ab} and A_{le} Flor (1946, p. 350) argues "since Abyssinian is susceptible to a number of North American races to which Leona is resistant, these two varieties possess different rust-conditioning factors. In all instances analyzable from these crosses as well as from the hybrid of race 6 with race 24 (Flor 1942) there is for each rust-conditioning factor in the host a factor for pathogenicity in the rust organism. Consequently it is probable that separate but closely linked factors condition pathogenicity on Abyssinian and Leona."

(ii) The second type of evidence consisted of disturbed F_2 joint segregations. On this basis, Flor classified A_{le} as linked with $A_{tpb}{}^1$, A_{ak} with $a_{bu}{}^{1-1}$, A_{ab} with $A_{bu}{}^1$, and A_k with A_{ir} (Table 7). Assessment of Flor's evidence and his interpretation is complicated by several sources of confusion. Difficulties of classification were

TABLE 6

FLOR'S (1946) EVIDENCE FOR COMPLETE LINKAGE BETWEEN PATHOGEN GENES AFFECTING HOST REACTION

I = Immune, R = resistant, S = susceptible, A = dominant gene conferring avirulence of pathogen on host indicated by subscript

Linkage Group	Host Variety Testers	Genes Segregating	Cross							
			Race 22 × Race 24		Race 6 × Race 22					
					Hybrid A		Hybrid B		Hybrid C	
			Reaction	Observed Frequency	Reaction	Observed Frequency	Reaction	Observed Frequency	Reaction	Observed Frequency
1	Akmolinsk Abyssinian Leona	A_{ak} * A_{ab} A_{le}	R : S I : S R : S	110 : 23	S R : S R : S	74 57 : 17	R : S I : S R : S	14 : 2	R : S I : S —	7 : 1
2	Pale Blue Crimped Kenya	A_p * A_k	R— : S R : S	96 : 37	R— : S R : S	59 : 15	R— : S R : S	12 : 4	R— : S R : S	8 : 0

* Groups of genes assumed by Flor to be linked.

also encountered. For example, distinction is made between "susceptible" and "less susceptible" in the reactions of F_2 cultures of race 22 × race 24 inoculated on to Buda to identify the gene, $a_{bu}{}^{1-1}$, in race 24 conditioning "moderate virulence". (Table 7).

There is an important difference between Flor's evidence for close linkage between genes in the pathogen and that used in support of his allelic series in the host. Whilst he admits that the failure of recombinant classes to appear could be evidence of allelism or close linkage between host genes, he does no more than infer that genes in the pathogen claimed to be "inherited as a unit on a single-factorial basis" cannot be allelic. Yet this seems to be the underlying reason for his adoption of a gene notation in the pathogen (Tables 6 and 7) unrelated to that adopted in the host.

But closeness of linkage between pathogen genes is dependent on the chance of a recombinant F_2 culture occurring at our accepted level of probability in the size of families Flor used. This chance is here $(2p - p^2)/2$, where p is the recombination fraction, since A_{ak}, A_{ab}, and A_{le}, and A_p and A_k enter crosses in coupling phase. The upper limits of the value of p for the F_2 families of race 22 × race 24 and hybrid A of race 6 × race 22 are 2·8 per cent. or higher.

Furthermore, the argument that Flor's "closely linked" pathogen genes could not be identical is not exclusive. Mere differences in reaction from one host "tester" to another may be equally well ascribed to differences in response of the same pathogen gene to the different host environments. Such differences, for example, do not justify the conclusion that the genes controlling virulence of race 24 to Akmolinsk and avirulence of the same race to Abyssinian are different, whilst genes giving such control to say Williston Golden and Williston Brown are identical.

In addition, Flor claims that there is a gene correspondence between host and pathogen, and, on this basis, differentiates between such genes as A_{ab} and A_{le}. But these same data are employed to develop the concept of gene correspondence and such gene correspondence is not complete. Flor's (1947) suggested genotypes for the host testers do not correspond, in every "variety", in the genes for "immunity" that they possess, to the genes for "avirulence" to that same variety shown to be present in the pathogen race (Flor 1946). For example, it is difficult to fit the genes concerned in Tammes Pale Blue into this concept, whilst the special case of a dominant gene for "virulence" in the pathogen appears to have no corresponding special case in the host varieties Williston Golden or Williston Brown. Flor (1954) suggests that correspondence is not always complete pending further tests, especially in new environments; this may be so, but it seems curious, considering the overwhelmingly greater volume of work carried out on host gene identification that there are pathogen genes identified for which corresponding host genes do not appear to have been identified.

A second unsatisfactory feature of the evidence relates to linkages based on disturbed F_2 joint segregations. Eight segregations (Table 7) are concerned. Two are shown to give disturbances insufficient to accept as serious without further data in support. It may be significant that these are the only ones involving F_2 cultures of race 22 × race 24. The remaining six segregations are derived from crosses between race 6 and race 22 which as Flor (1946) admits, are unsatisfactory because an in-

TABLE 7

FLOR'S (1946) EVIDENCE FOR LINKAGE BETWEEN PATHOGEN GENES AFFECTING HOST RESISTANCE

I = Immune, R = resistant, S = susceptible, R/SR = resistant or semi-resistant, R/S = resistant or moderately susceptible, A = dominant gene conferring avirulence of pathogen on host indicated by subscript

Linkage Group	Host Variety Testers	Genes Segregating	Cross	F_2 Segregation							Theoretical F_2 Ratio	χ^2 Values*				Upper Limit of Recombination
				Reaction and Numbers						Total		Total	Single Gene	Single or Duplicate Gene	Linkage	
1	Leona	A_{le} †	Race 6 × race 22	R	S	R	S									
	Tammes Pale Blue	A_{tpb}^{1} A_{tpb}		I	I	S	S						R : S	I : S		
			Hybrid A	56	12	0	5			73	45 : 15 : 3 : 1	$18.436_{(3)}$	0·114	0·045	17·874	Free
1	Akmolinsk	A_{ak} †	Race 22 × race 24	R	R	S	S									
	Buda	a_{bu}^{1-1}		S	S—	S	S—						R : S	S : S—		
				73	37	21	2			133	9 : 3 : 3 : 1	$11{\cdot}296_{(3)}$	4·213	1·326	5.757	Free
1	Abyssinian	A_{ab} †	Race 6 × race 22	R	S	R	S									
	Buda	A_{bu}^{1} A_{bu}		R/SR	R/SR	S	S						R/SR	R : S		
			Hybrid A	57	9	0	8			74	45 : 15 : 3 : 1	$47{\cdot}99_{(3)}$	2·63	0·16	45·20	Free
			Hybrid B	13	0	1	2			16	45 : 15 : 3 : 1					
			Hybrid C	7	0	1	1			9	45 : 15 : 3 : 1					
2	Kenya	A_k †	Race 22 × race 24	R	R	S	R	S	S							
	Italian Roma	A_{ir} A_{bg}		I	R/S—	I	S	R/S—	S							
				72	21	25	3	6	6	133	36 : 9 : 12 : 3 : 3 : 1	$9{\cdot}48_{(5)}$	—	—	—	—
			Race 6 × race 22													
			Hybrid A	45	0	11	5	0	13	74	24 : 3 : 8 : 5 : 3 : 5	$15{\cdot}71_{(5)}$	—	—	—	—
			Hybrid B	11	0	4	0	0	1	16	24 : 3 : 8 : 5 : 3 : 5	—	—	—	—	—

* Value of χ^2 at 5 per cent. probability = $3{\cdot}841$, $7{\cdot}815_{(3)}$, $11{\cdot}070_{(5)}$.

† Genes assumed by Flor to be linked.

sufficient number of cultures of each hybrid was tested to warrant an analysis, and the parental gametes of race 6 were heterogeneous. Even so, only two of them, both hybrid A cultures, show serious disturbances due to linkage, and their recombination values range from 0-50 per cent. at a 95 per cent. level of probability (Table 7).

VI. Host and Pathogen Gene Correspondence

Flor (1947, 1954) develops the idea of specificity of reaction between host and parasite to the point where the effects of one dominant gene in the host are exclusively specific to the effects of one dominant gene in the pathogen. For example, A_{ak}, A_{ab}, and A_{le} must be separate in their specificities though they segregate together (Table 6). The evidence is insufficient to support this extreme interpretation, which is not necessary for the basic usefulness of the concept in breeding for disease resistance.

Again, Flor's (1946) conclusion that specificity between the effects of host and pathogen genes ensures that pathogen strains arising through hybridization will not attack host strains previously resistant to both parental strains of the pathogen, is vulnerable. If host resistance depends upon dominant genes in the host (A and B) and parasite (Γ^A and Δ^B), two parasite varieties, $\Gamma^A\delta^B$ and $\gamma^A\Delta^B$, could be avirulent to host AB, the first because of the Γ^A-A reaction and the second because of the Δ^B-B reaction. A cross between the two parasites could yield two sorts of recombinants, $\Gamma^A\Delta^B$ and $\gamma^A\delta^B$, the second of which should be virulent towards the host AB. The fact, for example, that "Bombay, immune from races 6 and 22, was immune from the 98 F_2 cultures of this cross" could be due, as Flor suggests, to races both 6 and 22 being homozygous for a dominant gene conferring avirulence when reacting with a dominant gene for which Bombay is homozygous, conferring immunity: but if the two pathogens differed in their dominant genes for avirulence, then it could also be ascribed to the chance that 98 F_2 cultures failed to produce the necessary recombinant to permit virulence, either due to close linkage between the pathogen genes or to even more than two genes being concerned in the reaction. Three such independent genes segregating among 98 F_2 pathogen cultures would be sufficient to lower the chance of recovering the recombinant to approximately 0·2. The presence of additional dominant genes in Bombay, reacting with the pathogen but not yet identified, is a further possibility.

Flor (1947) tried to carry the concept of gene correspondence further and he examined the correspondence of "linkage groups" in the host and in the pathogen. In this connection, Flor apparently uses the term "linkage" to cover allelism and suggests that the linkage groups in the pathogen "transcend" those in the host. Presumably this means that members of a single linkage group in the pathogen correspond to genes belonging to more than one linkage group in the host. If this is the meaning intended, the case Flor (1947) actually quotes (see Section II (vii)) is based on very slender evidence (Tables 6 and 7). Furthermore, cases of members within each of Flor's L, M, and N alleles corresponding to different linkage groups of the pathogen can be quoted (see Flor 1946 and 1947). In any case, this deals with correspondence between groups not yet established.

VII. Discussion

Though this analysis shows that Flor's work establishes much less than is claimed, yet it is the most precise of its kind published and it does lead to a number of important suggestions.

The simplest hypothesis to account for the host-parasite reaction which is consistent with the genetical data is that this reaction is due to an interaction between a host substance and a pathogen substance, each the product of the respective host and pathogen gene. It is to this relationship that Flor (1947) seems to be referring in his idea of the correspondence between host and pathogen genes.

However, very little critical evidence of such a one to one relationship exists. Furthermore, we know that a number of other factors may affect the host-pathogen reaction though we do not know how or to what degree. The extent to which the rest of the genotype affects the expression of a particular gene is not known, though it is known that it can dominate the effect of a single gene. Environment, especially temperature, can manifestly alter the host-pathogen reaction though no carefully controlled measurements have been reported. Nor is it certainly known whether different strains inoculated on a host, either simultaneously or successively, interact, either physiologically or genetically. Finally, resolution of the genes concerned with disease resistance into their simplest units, for example by tests of recombination, has not been attempted, though it has obvious relevance to plant breeding. These considerations suggest promising fields of enquiry into the nature and inheritance of disease resistance.

The weakness of Flor's evidence shows the need for more critical tests for allelism and linkage between both host genes and pathogen genes. Figure 1 shows clearly that such tests are most critical when F_1 hybrids containing a pair of dominant genes conferring immunity are backcrossed to a universally susceptible host strain possessing both genes in the recessive state. The genes so tested, one from each parent incorporated in the F_1, are necessarily in the repulsion phase, so that the amount of information per plant grown is increased by a factor of $2/p$, where p is the recombination fraction, over that obtained in tests with F_2 families (Mather 1936): for the values of p less than 0·1, i.e., those in which we are interested, the difference in efficiency is overwhelming. Figure 1 shows that a family of about 300-400 backcross plants may test for values of p as low as 0·01. Kerr (1952) reports a number of conclusions involving host genes, added to Flor's allelic series, claimed to be based on this sort of backcross data, as yet unpublished. Flor (1954) has used backcrossing to build up "a reservoir of agronomically satisfactory" strains, each containing a single dominant host gene for immunity or resistance, but no segregations suitable for testing linkage have been reported.

In considering the suggestion that host genes are linked, rather than allelic, it may seem curious that all varieties so far tested carry only one locus of a linkage group as dominant.

This condition may be only apparent due to the present status of tests for allelism. None of Flor's reported work shows a test involving F_2 progeny of a cross between a host immune to both of any two pathogen strains and another host susceptible to the same two strains. The typical case concerns two dominant genes,

A and B, one conferring immunity to one pathogen and the other to a second pathogen. The possible genotypes are AB, Ab, aB and ab, between which six different crosses are possible, $AB \times Ab$, $AB \times aB$, $Ab \times ab$, $aB \times ab$, $Ab \times aB$, and $AB \times ab$. Only the last two can detect linkage between A and B, but Flor has never reported a cross of the last type with A and B in coupling phase crossed to a universal susceptible. Some of these genes already identified may, with more critical testing, prove to be two linked genes in coupling phase. Such a group of two genes (AB) tested against a third (C), the latter in repulsion phase with respect to the group (abC/ABc), may be expected to maintain its group unbroken, and either segregate or show allelism with respect to the third gene at Flor's level of testing, even though tests in repulsion are less sensitive than those in coupling phase. The chance of recovering a recombinant in repulsion is $p^2/4$ as against $(2p-p^2)/2$ in coupling phase, where p is the recombination fraction. This being so, linkage in coupling phase has not been found according to the data published so far. An added difficulty in trying to decide whether units of segregation are compound is that we cannot be certain that a phenotype will be the sum of its constituents.

Flor's publications for many other reasons have gained for him a proper recognition in plant breeding work but, in the process, the premises underlying his tests for allelism have become a generally accepted theory, especially in Australia (Kerr 1952; Finlay 1953; Pugsley and Carter 1953; Carter 1954).

VIII. ACKNOWLEDGMENTS

Professor D. G. Catcheside aroused my curiosity on many of the premises underlying theory and practice in modern plant breeding and has exhibited patience in all the ensuing discussions.

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IX. REFERENCES

BIFFEN, R. H. (1907).—Studies in the inheritance of disease resistance. *J. Agric. Sci.* **2**: 109-28.

CARTER, M. V. (1954).—Additional genes in *Triticum vulgare* for resistance to *Erysiphe graminis tritici*. *Aust. J. Biol. Sci.* **7**: 411-14.

FINLAY, K. W. (1953).—Inheritance of spotted wilt resistance in the tomato. II. Five genes controlling spotted wilt resistance in four tomato types. *Aust. J. Biol. Sci.* **6**: 153-63.

FLOR, H. H. (1935).—Physiologic specialization of *Melampsora lini* on *Linum usitatissimum*. *J. Agric. Res.* **51**: 819-37.

FLOR, H. H. (1940).—New physiologic races of flax rust. *J. Agric. Res.* **60**: 575-91.

FLOR, H. H. (1941).—Inheritance of rust reaction in a cross between the flax varieties Buda and J.W.S. *J. Agric. Res.* **63**: 369-88.

FLOR, H. H. (1942).—Inheritance of pathogenicity in *Melampsora lini*. *Phytopathology* **32**: 653-69.

FLOR, H. H. (1945).—Analytical key for the identification of physiologic races of *Melampsora lini*. Unpublished mimeograph issued by the U.S.D.A. and North Dakota Agric. Expt. Station.

FLOR, H. H. (1946).—Genetics of pathogenicity in *Melampsora lini*. *J. Agric. Res.* **73**: 335-57.

FLOR, H. H. (1947).—Inheritance of reaction to rust in flax. *J. Agric. Res.* **74**: 241-62.

FLOR, H. H. (1954).—Seed-flax improvement. III. Flax rust. *Advanc. Agron.* **6**: 152-61.

HENRY, A. W. (1930).—Inheritance of immunity from flax rust. *Phytopathology* **20**: 707-21.

KERR, H. B. (1952).—Rust resistance in linseed. *Nature* **169**: 159.

MATHER, K. (1936).—Types of linkage and their value. *Ann. Eugen. Camb.* **7**: 251-64.

MYERS, W. M. (1937).—The nature and interaction of genes conditioning reaction to rust in flax. *J. Agric. Res.* **55**: 631-66.

PUGSLEY, A. T., and CARTER, M. V. (1953).—The resistance of twelve varieties of *Triticum vulgare* to *Erysiphe graminis tritici*. *Aust. J. Biol. Sci.* **6**: 335-46.

STACKMAN, E. C., and PIEMEISEL, F. J. (1917).—Biologic forms of *Puccinia graminis* on cereals and grasses. *J. Agric. Res.* **10**: 429-95.

HOST PATHOGEN RELATIONS IN POWDERY MILDEW OF BARLEY

II. CHANGES IN RESPIRATORY PATTERN

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Summary

The histological nature of resistance of barley seedlings to infection by *Erysiphe graminis* var. *hordei* is well understood. Infection by this obligate parasite causes marked biochemical changes in the host. These changes are expressed in an increased respiratory rate. The pattern of respiratory change is different and typical for the varietal types tested and may be correlated with the observed histological changes. This increased respiration may well be due to the formation, either by the pathogen or by the host pathogen combination, of an agent which uncouples directly or indirectly the concomitant phosphorylations normally limiting the respiratory rate.

I. INTRODUCTION

Wheat seedlings infected with *Erysiphe graminis* var. *triticii* have been shown (Allen and Goddard 1938) to respire at a rate much higher than that of non-infected wheat of the same age. This increase in respiration was shown to be localized in the mesophyll tissue although it is not necessarily in immediate contact with the invading ectoparasite. Several possible explanations of this changed respiratory pattern can be proffered (Allen 1953), including the presence of an uncoupling agent, produced either directly by the pathogen or by the host-pathogen combination. It is established that the factor frequently limiting respiration is the rate of concomitant phosphorylations (Potter and Recknagel 1951; Lardy and Wellman 1952). There is one physiological respiratory rise occurring in higher plants which has been explained on the basis of the formation of an uncoupling agent. This is the climacteric rise in respiration which accompanies the ripening of some fruits (Millerd, Bonner, and Biale 1953).

Histological studies on host-pathogen relations in powdery mildew of barley (White and Baker 1954) indicate that resistance in barley to infection by *E. graminis* var. *hordei* Marchal is a function of the rapidity and degree of mesophyll collapse rather than being the result of death of epidermal cells or aborted development of haustoria. White and Baker (1954) found that within 30 hr after inoculation an haustorium was formed in each infection court of all types of barley. In highly resistant barley varieties, mycelial growth was restricted, haustoria were fewer, and mesophyll collapse more rapid (30-48 hr after inoculation) than in resistant, semi-resistant, and susceptible varieties. In these three variety groupings during the first 54 hr after inoculation, the amount of mycelial growth and the number of haustoria formed per infection court were the same. There was no collapse of mesophyll cells during this period. In the resistant hosts, mesophyll collapse commenced at 54-72 hr after inoculation. After cell collapse had commenced haustoria and mycelial development were

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restricted. In semi-resistant hosts, there was a rapid collapse of mesophyll cells 72-90 hr after inoculation. Coincident with this collapse, as in resistant varieties, there was a restriction of haustoria and mycelial development. In the susceptible varieties there was unrestricted development of haustoria and mycelia during the first 90 hr and no mesophyll collapse.

The purpose of this investigation was to try to establish whether the premise of the formation of an uncoupling agent could be correlated with these histological findings.

II. MATERIALS AND METHODS

The barley varieties investigated were B49 "Kinver", susceptible; "Cape", susceptible; B167, resistant; B69, semi-resistant; and B278, highly resistant to the pathogen, *E. graminis* var. *hordei* Marchal. The plants were grown and inoculated 7 days after planting according to the procedure of White and Baker (1954), the greenhouse temperature range was 25-30°C unless otherwise stated.

TABLE 1

OXYGEN CONSUMPTION OF NON-INFECTED TISSUE

Variety	Age (days after planting)	Experiment No.						
		1	2	3	4	1	2	3
		Oxygen Consumed per 60 Min (μl)				Oxygen Consumed per 120 Min (μl)		
Kinver	8	64	64	59		117	119	111
Cape	8	48	45	46	51			

Respiration studies were conducted at 30°C using the standard Warburg technique. The vessels were covered with black cloth to eliminate photosynthesis. The leaves were cut in 1-cm lengths. In each experimental vessel 200-mg aliquots were floated in 5×10^{-2}M potassium dihydrogenphosphate. The vessels were allowed to equilibrate for 15 min, the taps closed, and readings taken at 15-min intervals. All figures for gas exchanges represent the mean of two or three samples.

III. RESULTS

It was first necessary to ascertain whether infection of barley by powdery mildew affected the leaf respiration in a manner similar to that observed with infected wheat (Allen and Goddard 1938). A number of experiments were conducted to characterize barley leaf respiration and to establish optimal conditions for respiration measurements.

(*a*) *Examination of Respiration of Barley Leaves*

(i) *Magnitude of Respiration.*—Two hundred mg of non-infected leaf tissue, which showed an oxygen consumption of *c.* 60 μl/hr, was used in all experiments.

TABLE 2

EFFECT OF ADDED SUBSTRATE ON THE RESPIRATORY RATE OF BARLEY LEAF, VARIETY B69, AGE 9 DAYS*

Substrate	Concentration (M)	Oxygen Consumed per 60 Min (μl)	Oxygen Consumed per 120 Min (μl)
—	—	143	268
Sucrose	10^{-2}	139	274
Glucose	10^{-2}	144	256
Citrate	10^{-2}	138	275
Succinate	10^{-2}	139	245

* The tissue used in this experiment was grown at a greenhouse temperature of 18°C. The higher respiration rate observed is due to the fact that the tissue is relatively young. Similar results have been obtained with tissue grown at 25-30°C.

(ii) *Variation in Oxygen Consumption of a Number of Samples Taken from One Batch of Seedlings.*—Replicate measurements of oxygen consumption of a number of samples showed good agreement as is seen in Table 1.

TABLE 3

EFFECT OF 2,4-DINITROPHENOL ON THE RESPIRATION OF BARLEY LEAF, VARIETY B69, AGE 9 DAYS

2,4-Dinitrophenol Concentration (M)	Oxygen Consumed per 60 Min (μl)	Increase (%)
—	90	—
10^{-3}	85	−6
3×10^{-4}	139	54
10^{-4}	114	27
3×10^{-5}	110	22
10^{-5}	91	1
3×10^{-6}	84	−7

(iii) *Effect of pH.*—Respiration studies were made at pH 4·5, 5·5, 6·0, and 7·0. No significant differences in oxygen consumption were observed. Vacuum infiltration of the buffers did not alter this finding.

(iv) *Effect of Added Substrates on the Rate of Respiration*.—Since the rate of respiration of tissues may be limited by the availability of substrate, it is interesting to note that the rate of respiration was unaffected by added substrates (Table 2). Vacuum infiltration of the substrates did not alter this result. The fact that added substrates caused only very small increases in the respiration of leaf tissue indicated that, in the non-infected leaf, substrate was non-limiting.

(v) *The Effect of Dinitrophenol on Respiration Rate*.—That the leaf respiration was controlled however by coupled phosphorylation may be shown by the effect of 2,4-dinitrophenol (DNP). DNP at 3×10^{-4}M concentration caused a 54 per cent. increase in respiratory rate (Table 3).

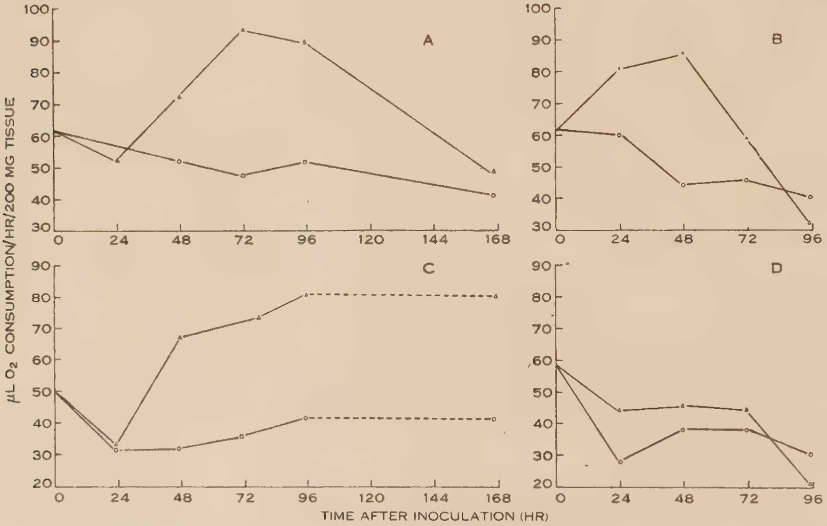

Fig. 1.—Comparison of respiration of non-infected and infected barley leaf. *A*, strain B69, semi-resistant. *B*, strain 278, highly resistant. *C*, strain B49, susceptible. *D*, strain B167, resistant. ○ Non-infected tissue. △ Infected tissue. - - - - From another experiment (see Fig. 1*C*).

(*b*) *Increase in Respiration of Barley Leaf after Inoculation with* E. graminis *var.* hordei

(i) *Respiration of Non-infected and Infected Barley Leaves*.—Normally the rate of respiration of barley leaf tissue, expressed on a wet weight basis, showed a gradual decrease with increasing age of the tissue. However, as is shown in Figure 1*A*, the rate of barley leaf respiration increased markedly after inoculation with *E. graminis* var. *hordei*. The increased oxygen consumption is accompanied by a corresponding increased carbon dioxide evolution (Table 4) indicating an increase in true respiration after infection. The marked increase in respiration was observed within 48 hr although in the semi-resistant strain B69 no histological evidence of the pathogen can be detected until 72 hr after inoculation.

The increased respiration observed would actually be much larger if the results were expressed on a surface area basis, since 200 mg fresh weight of non-infected

B69 tissue corresponded to 11·37 cm^2 (mean of three determinations) and 200 mg fresh weight of infected tissue corresponded to 10·05 cm^2 (mean of three determinations).

TABLE 4

COMPARISON OF OXYGEN CONSUMPTION AND CARBON DIOXIDE EVOLUTION OF NON-INFECTED AND INFECTED BARLEY LEAF TISSUE VARIETY B69, AGE 12 DAYS, 96 HR AFTER INOCULATION

Tissue Type	Oxygen Consumed per 60 Min (μl)	Carbon Dioxide Evolved per 60 Min (μl)
Non-infected	66	78
Infected	126	139

The tissue used for the experiments recorded in Tables 4 and 5 was grown in May 1955 with a greenhouse temperature of approximately 18°C. The lower growing temperature makes it necessary to inoculate at a later stage (8 days in these cases). Growth of both seedling and fungus is slower, but the relationship of respiration and histological changes remains the same: it is merely spread out over a longer period of time.

(ii) *Localization of Increased Respiration*.—The increased respiration observed on infection is not due to the respiration of the attached fungus. The fungus may be removed without apparent injury of the host by brushing with a camel hair brush (Allen and Goddard 1938). After B69 infected tissue is so treated only a few haustoria remain, yet the respiration of the treated infected tissue is almost as high as that of untreated infected tissue (Table 5). It was not possible to extend these studies on the localization of increased respiration as was done by Allen and Goddard as it proved extremely difficult to strip off undamaged mesophyll-free epidermis from barley leaf.

TABLE 5

COMPARISON OF RESPIRATORY RATES OF NORMAL AND MILDEWED BARLEY LEAF TISSUES VARIETY B69, AGE 12 DAYS

Tissue Type	Oxygen Consumed per 60 Min per 200 mg Tissue (μl)		
	Expt. 1	Expt. 2	Expt. 3
Non-infected leaf	68	65	
Mildewed leaf	124	128	
Same leaf, mildew removed	110	112	116

(c) *Studies on Changes in Leaf Respiration of Four Varieties of Barley after Inoculation with* E. graminis *var.* hordei

The differential response of barley varieties to infection may be clearly demonstrated histologically. It is of interest, therefore, to determine if varietal differences are also expressed in the respiratory patterns following infection.

To study this problem various barley varieties were planted, and after 7 days one-half the seedlings were inoculated. The respiration of the non-infected and infected tissues was measured at 24-hr intervals. The results are shown in Figures 1*A*-*D*. All the barley varieties investigated have been tested at least twice, and no experiments conducted showed any variation in the curve characteristic for a particular variety although the absolute increases differed.

As is shown graphically the presence of the pathogen resulted in increased respiration and in all cases this increase precedes any detectable histological change. The changed respiratory pattern is, however, different and typical for each barley variety tested. The respiration increase occurred earliest (Fig. 1*B*) in the highly resistant strain B278 where the mesophyll tissue is most sensitive and collapses most rapidly. Infection of the semi-resistant strain (B69) resulted (Fig. 1*A*) in increased rate of respiration 48 hr after inoculation, which continued high until collapse of the cells and cessation of parasite growth. In the susceptible strain (B49), the onset of increased respiration (Fig. 1*C*) was delayed until 48 hr and the increase continued gradually as more cells became invaded by the parasite. The resistant strain B167 shows a cytological picture rather different from a state intermediate between semi-resistant and susceptible. It is of interest therefore that this was also true of the respiratory picture on infection (Fig. 1*D*). The histological picture shows odd cells collapsing from 30 hr after inoculation with the main cell collapse being observed at 72 hr. A respiratory rise occurred 24 hr after inoculation and a small increased respiration was maintained as long as fungal development continued. This relatively flat curve of increased respiration may be interpreted as being the sum of two processes—the increased respiration of progressively affected cells together with a trend towards normal respiration as these cells gradually collapse.

IV. DISCUSSION

In highly resistant barley strains which will not tolerate the growth of the parasite *E. graminis* var. *hordei*, there was a very rapid biochemical response to the presence of the ectoparasite. A particularly rapid rise in respiration occurred followed by early collapse of the affected cells and a return of the respiratory rate to normal levels. In the susceptible strain, the onset of the response to the pathogen was less dramatic and the increased respiration continued parallel with fungal growth. Resistant and semi-resistant barley strains showed on infection respiratory patterns in accord with their recorded cytological behaviour. In these two the respiratory pattern was correlated with the time and extent of cell collapse. In all instances, increased respiration occurred in advance of cell collapse. Complete cell collapse and cessation of fungal growth, as occurs in highly resistant strains, resulted in a return to normal of the respiratory rate. In resistant strains where cell collapse takes place over a long period of time as mesophyll tissue becomes progressively affected by the fungus, the small increased respiration was maintained. This respiratory pattern can be explained as being the resultant of two opposing processes. As cells collapse there was a tendency of the respiratory rate to return to normal and this was counteracted by the increased respiration resulting from continuing fungal growth which affects further leaf cells.

The nature of this increased respiration can be established. The increase cannot be due to the respiration of the attached fungus, since infected tissue with the fungus removed respired at almost the same rate as tissue with the fungus attached. That the rate of respiration of non-infected barley leaf tissue was not limited by the level of substrate was demonstrated. Thus the increased respiration cannot be explained by the suggestion that, on infection, more substrate was somehow made available to the cells. However, from the effects of DNP on barley leaf respiration it is apparent that respiratory rate was limited by the capacity of the phosphorylative system. When the respiration was artificially uncoupled from its concomitant phosphorylations, the rate was increased approximately 60 per cent.

The removal *in vivo* of the limitation of the phosphorylative system can be achieved in two very different ways. Firstly, either the rate of turnover of the phosphate acceptor, adenosine diphosphate (ADP), may be increased by an increased rate of synthetic reactions utilizing adenosine triphosphate (ATP), or the amount of ADP could be increased; both would lead to the formation of increased amounts of ATP and thus enable increased rates of synthesis. Secondly, either the rate of turnover of ADP could be increased by the liberation or formation of ATPase which would break down ATP immediately it was formed, or an agent might be formed which would uncouple the phosphorylative processes prior to the formation of ATP. The two uncoupling procedures are degenerative processes and would lead to disorganization and finally collapse of the cell.

The respiration of non-infected barley leaf is limited by the capacity of its phosphorylative system. The increased respiration observed on infection precedes any detectable histological changes but is later accompanied in highly resistant, semi-resistant, and resistant strains by marked degenerative changes leading finally to collapse of affected cells. In an infected susceptible strain, the marked increased respiratory rate is maintained for a long period of time but is not accompanied by cell collapse. The presence of the pathogen does, however, cause marked changes in the growth pattern—growth is stunted and limited, crop yield (if the plants survive long enough) is reduced, and the leaves die prematurely. All these are degenerative changes and may be interpreted, at least in part, by the hypothesis that here too uncoupling occurs but in this instance it is partial rather than absolute. This uncoupling could occur either directly by the formation of an agent with properties like DNP, or indirectly by the formation of a toxic agent causing a disorganization within the cell leading to the liberation of ATPase.

It would appear then that all the recorded changes, both biochemical and histological, may be explained by the postulate that the presence of the pathogen or the host-pathogen combination results in the formation of an agent which, either directly or indirectly, uncouples barley leaf respiration from its concomitant phosphorylations. Investigations are planned to elucidate the nature of this agent.

V. Acknowledgments

The authors are indebted to Dr. N. H. White, Faculty of Agriculture, University of Sydney, for the suggestion that, on infection, varietal differences in barley might find expression in differential respiratory behaviour; and to Dr. E. P. Baker, Faculty of Agriculture, University of Sydney, for providing the plant materials. This work has been supported by a University of Sydney Research Grant.

VI. References

Allen, P. J. (1953).—Toxins and tissue respiration. *Phytophathology* **43**: 221-9.

Allen, P. J., and Goddard, D. R. (1938).—A respiratory study of powdery mildew of wheat. *Amer. J. Bot.* **25**: 613-21.

Lardy, H. A., and Wellman, H. (1952).—Oxidative phosphorylations: role of inorganic phosphate and acceptor systems in control of metabolic rates. *J. Biol. Chem.* **195**: 215-24.

Millerd, A., Bonner, J., and Biale, J. B. (1953).—The climacteric rise in fruit respiration as controlled by phosphorylative coupling. *Plant Physiol.* **28**: 521-31.

Potter, V. R., and Recknagel, R. O. (1951).—The regulation of the rate of oxidation in rat-liver mitochondria. In "Phosphorus Metabolism. Symposium on the Role of Phosphorus in the Metabolism of Plants and Animals." Vol. I, pp. 377-85. (Ed. W. D. McElroy and B. Glass.) (The Johns Hopkins Press: Baltimore.)

White, N. H., and Baker, E. P. (1954).—Host-pathogen relations in powdery mildew of barley. 1. Histology of tissue reactions. *Phytopathology* **44**: 657-62.

SOME INTERACTIONS BETWEEN PLANT ROOTS AND PATHOGENIC SOIL FUNGI

By A. Kerr*

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Summary

A "Cellophane" bag technique is described which enables some chemical interactions between organisms to be studied *in situ*.

Both *Pellicularia filamentosa* and *Sclerotinia homeocarpa* affect a wide range of plants. *P. filamentosa* causes typical damping-off of seedlings whereas *S. homeocarpa* causes a severe stunting without penetrating the plant tissue. It was shown by the "Cellophane" bag technique that these fungi excrete substances toxic to the plants, and also that the roots of lettuce and radish seedlings stimulate the growth of *P. filamentosa* but the roots of tomato seedlings do not. Preliminary investigations on the relation of the root excretions to infection and susceptibility of seedlings are described.

I. Introduction

The interactions between plant hosts and fungal pathogens have been studied intensively by workers in many countries, and much is now known about the post-penetration stages of infection; the pre-penetration stage of infection, however, has been relatively neglected although it is well established that the host plant can influence the pathogen before penetration takes place. W. Brown (1922) showed that the germination of spores of *Botrytis cinerea* was stimulated by diffusions from damaged plant tissues, and R. Brown (1946) has reviewed the literature on the influence of root excretions on the germination of spores of pathogenic fungi. The reverse effect of pathogen on host has also been noted by some workers. Müller (1924) observed that necrotic spots appeared on potato roots prior to penetration by hyphae of *Pellicularia filamentosa*, and Lebeau and Dickson (1953) have shown that an unidentified snow-mould pathogen liberates hydrogen cyanide in toxic quantities.

In the course of work on two lawn diseases, brown patch and dollar spot, caused by *P. filamentosa* (Pat) Rogers and *Sclerotinia homeocarpa* Bennett respectively, some interesting results were obtained on the mutual influence of host and pathogen prior to penetration. The present paper reports these results and discusses their significance.

II. Experimental

(*a*) *Pathogenicity of* P. filamentosa *and* S. homeocarpa *to Several Plants*

At least 230 plant species belonging to 66 families have been recorded as hosts of *P. filamentosa* (Braun 1930). Storey (1941) showed that the fungus exists as several strains, some of which have a wide host range while others are more specialized and may be restricted to hosts of a single family. The literature on physiological specialization of *P. filamentosa* has been reviewed by Kernkamp *et al.* (1952).

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S. homeocarpa has been recorded as causing disease only in grass lawns, but Montieth and Dahl (1932) state that the fungus may attack a wide range of weeds in lawns. In the present work, the pathogenicity of *P. filamentosa* and *S. homeocarpa* to several plants of economic importance was tested. *P. filamentosa* (Waite Institute strain St 3) was isolated from a lawn infected with brown patch, and *S. homeocarpa* from a golf green infected with dollar spot.

The plants tested were: *Raphanus sativus* L. (radish), *Lactuca sativa* L. (lettuce), *Beta vulgaris* L. (red beet), *Lycopersicon esculentum* Mill. (tomato), *Trifolium subterraneum* L. (subterranean clover), *Triticum vulgare* Vill. (wheat), *Pisum sativum* L. (pea), and *Phaseolus vulgaris* L. (bean).

TABLE 1

THE PATHOGENICITY OF P. FILAMENTOSA AND S. HOMEOCARPA TO SEVERAL PLANTS

Percentage of healthy, emerged seedlings from four jars

Plant	Control	*P. filamentosa*	*S. homeocarpa*	No. of Seeds Sown
Radish	81	1	0	80
Lettuce	83	4	4	80
Beet	130	1	1	80
Tomato	91	19	16	80
Subterranean clover	80	8	3	80
Pea	83	42	0	40
Bean	90	95	0	20
Wheat	93	83	75	40

Pathogenicity was measured by sowing seeds of the various test plants in inoculated and in uninoculated soil and counting the subsequent emergence of seedlings. To inoculate soil, the fungi were grown separately on sand-maize-meal for 10 days and then mixed thoroughly with unsterilized soil at the rate of 2 g inoculum to 100 g soil. Cylindrical glass jars, 9 cm deep and 9 cm wide were used as soil containers. Unless otherwise stated this method of soil inoculation was used throughout. There were four replicates of each treatment and the experiment was run in a constant temperature growth room at 22°C. The results were recorded after 10 days (Table 1).

The results indicate that *P. filamentosa* (strain St 3) has a wide host range. Most of the susceptible seedlings were attacked in the pre-emergence stage, while the few seedlings that did emerge were often diseased. Of the small-seeded, non-graminaceous plants tested, tomato appeared to be more resistant than the others. The emergence of beans was not affected by the fungus, which produced limited, dark brown lesions on the hypocotyls near soil level. The effect on peas was similar but they were more susceptible than beans, and the lesions sometimes completely encircled the hypocotyls causing the seedlings to collapse. Symptoms produced on wheat closely resembled the disease "sharp eye spot" described by Glynne and Ritchie (1943). There was very little damage to the roots of any of the plants tested.

Sclerotinia homeocarpa also appeared to have a wide host range but the effect of this fungus on the host was quite different to that caused by *P. filamentosa*. The seedlings which emerged were severely stunted (Plate 1) but when they were dug up, washed, and examined, there was no sign of penetration of plant tissue by the fungus. The non-emerged seedlings were then recovered from the soil and examined. Tomato, beet, radish, lettuce, subterranean clover, and wheat seedlings were all severely stunted, but showed no sign of fungal infection. Half of the bean seeds sown had not germinated while the others had produced radicles about ½ in. long. The tips of the radicles were brown and necrotic, but no mycelium could be found within the root tissue. Necrotic roots were washed thoroughly in sterile water and planted on tap water-agar; the presence of *S. homeocarpa* could not be demonstrated. Peas were even more severely affected than beans and germination had been arrested at a very early stage. The pea and bean seeds were soft, and microscopic examination showed that the cotyledons were heavily infected with bacteria and fungal hyphae. *S. homeocarpa* was readily isolated from these seeds.

(*b*) *Chemical Interactions between Hosts and Pathogens*

The previous experiment showed that the pathogenic activities of *P. filamentosa* (strain St 3) and *S. homeocarpa* were very different: *P. filamentosa* penetrated the tissues of many plants and caused pre-emergence or post-emergence damping-off of seedlings; *S. homeocarpa* also damaged a wide range of plant seedlings, but the effect of the fungus on all hosts except beans and peas was not direct parasitic attack, but appeared due to the production of a toxin which prevented or retarded growth.

Further studies of the interactions of hosts and fungi have been undertaken using a "Cellophane" bag technique to prevent physical contact between host and fungus and yet allow diffusion of chemicals from one to the other. Dobbs and Hinson (1953) have used "Cellophane" bags to study the behaviour of fungal spores in soil, and by this method were able to demonstrate a widespread fungistasis in soil that prevented the germination of spores of many soil fungi. In the present studies, seeds of various hosts were placed in "Cellophane" bags which were then part buried in soil inoculated with the appropriate fungus. "Cellophane" sheets, 25 μ thick, were boiled in water, as recommended by Dobbs and Hinson, to remove any coating material, and then made into bags 2 by 2·5 in. using adhesive "Cellophane" to seal them. Eight seeds of lettuce or radish were placed in the lower half of a "Cellophane" bag, and two bags of each plant were then partly buried in 400 g unsterilized soil in a glass jar. The soil had been inoculated previously with sand-maize-meal cultures of *P. filamentosa* or *S. homeocarpa* or left uninoculated. The soil containers were covered with clear plastic to prevent evaporation, and the experiment was carried out in quadruplicate in a constant temperature growth room at 22°C. After 3 days incubation, the bags were taken carefully from the soil and washed gently in water to remove adhering soil particles.

The seedlings from the bags buried in soil inoculated with *S. homeocarpa* were markedly stunted compared with the controls (Plate 2, Fig. 1). Some of the lettuce roots were necrotic but in no case had the fungus penetrated the "Cellophane". Stunted and necrotic seedlings were planted on tap water-agar and potato-dextrose-agar but *S. homeocarpa* did not grow from them. This confirms that *S. homeocarpa* produces a substance toxic to plants, and that the plants may be affected without the fungus penetrating them. The substance is soluble in water and is capable of diffusion through "Cellophane".

The "Cellophane" bags buried in soil inoculated with *P. filamentosa* were examined, and it was found that there was marked aggregation of hyphae on the outside of the "Cellophane" directly opposed to some of the roots of both radish and lettuce seedlings. Where aggregation had occurred the radish roots were blackened and the lettuce roots severely necrotic. The aggregated hyphae outside the bags were removed from the "Cellophane" by means of a needle and forceps, planted on tap water-agar, and were shown to be *P. filamentosa*.

Further experiments showed that the aggregation of *P. filamentosa* hyphae could be demonstrated more clearly if sterile, germinating seedlings (four per bag) were used instead of seed, and if the soil was left for 1 week after inoculation before the "Cellophane" bags were buried. If the bags were buried in the soil immediately after inoculation, there was dense hyphal growth over the "Cellophane" in the soil and stimulation of the fungus by the roots was sometimes obscured. Staining the bags with cotton blue in lactophenol, after removal from the soil, facilitated examination of the fungal hyphae.

Using this modified technique, tomato, lettuce, and radish seedlings were tested in unsterilized soil inoculated with *P. filamentosa* and in uninoculated soil, and four bags from each treatment were examined after 3, 5, and 7 days incubation. After 3 days incubation there was a marked aggregation of hyphae opposite the roots of lettuce and radish seedlings in inoculated soil (Plate 2, Fig. 2). There was no aggregation of fungal hyphae opposite tomato roots in inoculated soil or opposite the roots of the three test species in control soils. It was again observed that where aggregation had occurred, the roots of the plants were damaged and the seedlings were markedly stunted (Plate 3). The bags were opened and the "Cellophane" carefully examined under a microscope for hyphal penetration but none were found. Hyphae of *P. filamentosa* did not grow from affected seedlings which were planted on tap water-agar and potato-dextrose-agar. After 5 days incubation, aggregation of hyphae was more intensive and damage to seedlings more severe. Cultural studies showed that the fungus had penetrated four of the "Cellophane" bags, two containing radish and two containing lettuce seedlings, and that the seedlings in the bags were infected. After 7 days incubation, all bags containing radish or lettuce seedlings in inoculated soil were severely rotted and it was difficult to remove them from the soil without damage; the bags tore only where there was intensive hyphal aggregation. Those bags containing tomato seedlings in inoculated soil and those containing radish, lettuce, and tomato seedlings in uninoculated soil were intact. There was no aggregation opposite the hypocotyls of any seedlings of the three test plants.

The aggregation must have resulted from the diffusion of a substance, or substances, from the plant roots through the "Cellophane" to the soil, and this substance stimulated the growth of *P. filamentosa*. The damage to the plant roots prior to penetration of the "Cellophane" by the fungus, must have been caused by the diffusion of a substance from the aggregated hyphae through the "Cellophane" to the plant roots.

In inoculated soil the weakening of the bags containing radish and lettuce seedlings could have been caused by the physical penetration of the "Cellophane" by the fungal hyphae or by the cellulolytic activity of *P. filamentosa* or associated soil organisms. *P. filamentosa* has been classified by Garrett (1951) as a "sugar fungus", a term first used by Burges (1939) to denote those soil fungi that are unable to decompose either cellulose or lignin. Blair (1943) has demonstrated, however, that *P. filamentosa* is able to decompose cellulose, although much more slowly than typical cellulose decomposers, and Siu (1951) lists *P. filamentosa* as a cellulolytic fungus.

(*c*) *The Relation of Root Stimulation to Infection and Susceptibility of Seedlings*

Experiments with the "Cellophane" bags had indicated that growth of *P. filamentosa* over the "Cellophane" was denser when the bags were placed in the soil immediately after inoculation than when they were added 1 week later (see Section II (*b*)). It is unlikely that this difference was the result of a rapid decrease in the fungal population in the soil; a simpler explanation would be that shortly after inoculation the fungus was actively colonizing the soil, whereas after 1 week the process of colonization was largely completed, resulting in a decrease in the number of growing tips of the fungus. If infection of seedlings is dependent on a stimulation of the fungus before penetration, the artificial stimulus following soil inoculation might influence the number of seedlings infected. To test this, radish, lettuce, and tomato seeds were sown in soil (not in "Cellophane" bags) immediately after inoculation and 2 weeks after inoculation, and also in uninoculated soil.

Table 2

THE INFLUENCE OF TIME OF SOWING AFTER SOIL INOCULATION WITH P. FILAMENTOSA ON THE EMERGENCE OF SEEDLINGS

Percentage of healthy, emerged seedlings from four pots each sown with 20 seeds

Time of Sowing after Inoculation	Lettuce	Radish	Tomato
Not inoculated	78	71	83
Two weeks	0	1	51
Immediately	0	0	13

The results were recorded after 10 days incubation at 22°C. Table 2 shows that there was a much higher number of healthy tomato seedlings in the soil sown 2 weeks after inoculation than in the soil sown immediately after inoculation, but that the number of healthy radish and lettuce seedlings in the two soils was not significantly different. These results show that an increase in the activity of the fungus in the soil leads to an increase in the number of tomato seedlings infected; they do not indicate, however, whether the difference in emergence between tomato seedlings on the one hand and lettuce and radish seedlings on the other, is due to the latter being more susceptible, or to the stimulation of the fungus by their root excretions. If the second alternative is true, it is possible that by growing tomato plants in close association with radish and lettuce plants, stimulation of the fungus by the latter could influence the number of tomato seedlings infected. This was tested by sowing tomato seed in contact with radish and lettuce seed in soil inoculated 2 weeks previously, and in uninoculated soil. Seeds of the three plants were also sown separately. The emergence of tomato seedlings from inoculated soil was much higher when sown alone than when grown with radish and lettuce seedlings (Table 3), suggesting that stimulation of the pathogen by root excretions from the latter plants caused an increase in the number of infected tomato seedlings. Stimulation of the fungus resulting from infection of radish and lettuce seedlings may be an alternative explanation.

TABLE 3

THE EFFECT OF SOWING LETTUCE, RADISH, AND TOMATO SEEDS ALONE AND ALL TOGETHER IN SOIL INOCULATED WITH P. FILAMENTOSA

Percentage of healthy, emerged seedlings from four pots

	Method of Sowing	Lettuce	Radish	Tomato
Control	Alone	81	66	81
	All together	78	66	82
P. filamentosa	Alone	3	2	61
	All together	1	0	20

From these experiments a marked difference in the susceptibility of tomato seedlings and radish and lettuce seedlings was not demonstrated conclusively. The difficulty has been to distinguish between the effect of fungal activity as influenced by root excretions and true host susceptibility. The "Cellophane" bag experiments, however, had failed to demonstrate a stimulation of the fungus by the hypocotyls of any seedlings, and in an attempt to determine the true susceptibility of radish, lettuce, and tomato to *P. filamentosa* (strain St 3), a different method of inoculation was used. Seeds of the three plants were sown separately in uninoculated soil in eight glass jars, 20 seeds being sown in each. Immediately after seedling emergence, discs (6 mm in diameter) of potato-dextrose-agar inoculum were placed on the soil surface at the centre of half the jars, the remainder being left uninoculated, and all were covered with clear plastic. The fungus grew out radially from the inoculum over the surface of the soil. Although some hyphae probably grew through the soil, the effect of root stimulation must have been considerably reduced. 100, 96·7, and 39·6 per cent. of lettuce, radish, and tomato seedlings respectively, were diseased. This indicates that tomato seedlings are more resistant to infection by *P. filamentosa* than are lettuce and radish seedlings, although the possibility of a chemical stimulation by the hypocotyls of the susceptible seedlings cannot be entirely ruled out. There was a marked difference, however, between the appearance of the affected plants; the hypocotyls and cotyledons of infected lettuce seedlings became completely permeated by the fungus mycelium, whereas damage to the tomato seedlings was largely confined to the base of the hypocotyls at soil level. The appearance of radish seedlings was intermediate to that of lettuce and tomato seedlings; frequently the cotyledons and the upper part of the hypocotyls were infected. These observations support the belief that tomato seedlings are more resistant to infection by *P. filamentosa* than are lettuce and radish seedlings.

III. Discussion

Both *P. filamentosa* and *S. homeocarpa* would be classified by Gäumann (1950) as "necrobionts"; they "never feed directly on living plant substance because the toxic secretions of their hyphae kill the host cells in advance of them." When *S. homeocarpa* is inoculated into soil it grows very vigorously for a limited period, and in the process secretes a toxic substance in sufficient concentration to depress the growth of seedlings of several plant species which are not penetrated by the fungus as far as I have been able to discover. Most seedlings are not markedly damaged by the toxin, the exceptions being pea and bean seedlings, whose roots are severely damaged by the toxin. An effect similar to this has been demonstrated by Newton and Mayers (1935) for a strain of *P. filamentosa* on turnips and carrots. "Turnips are markedly stunted when grown on infected soil but exhibit no lesions or other symptoms." *P. filamentosa* (strain St 3) also excretes a toxin which may cause a necrosis of roots before the plant tissues are penetrated by the fungus. The production of toxins by both fungi was demonstrated conclusively by the "Cellophane" bag technique. This technique also showed that *P. filamentosa* (strain St 3) is stimulated by a substance excreted by the roots of radish and lettuce seedlings. This stimulation caused the hyphae of *P. filamentosa* to aggregate on the outside of the "Cellophane" bags directly opposed to the roots. Both stimulatory and toxic substances must be soluble in water and be capable of diffusion through thin "Cellophane".

Evidence was presented to show that the excretion of stimulatory substances by roots makes seedlings more liable to infection, and also that tomatoes which did not stimulate the fungus were less susceptible than radish and lettuce which did. This aspect of the work is being investigated further.

In the "Cellophane" bag experiments, fungal growth was not stimulated by the hypocotyls of any of the test plants, and yet when seedlings are not enclosed in "Cellophane" bags, *P. filamentosa* (strain St 3) attacks the hypocotyls and not the roots. A root does not have an impervious covering of cuticle and so presumably substances can diffuse into the soil much more readily from a root than from a hypocotyl. Either the hypocotyls did not excrete a stimulatory substance, or the substance did not diffuse through the "Cellophane" in sufficient quantity to stimulate growth. The contact between hypocotyl and "Cellophane" was never as close as between root and "Cellophane", as the cotyledons in their upward growth pushed apart the two sides of the bag, and this may have prevented the diffusion of substances from the hypocotyls to the surrounding soil. It is possible that when host and pathogen are in direct contact there is sufficient diffusion from the hypocotyls to influence the growth and behaviour of the fungus. If this is the case, the stimulation of the fungus by the hypocotyls might be much more important than stimulation by roots, and root excretions may be largely fortuitous due to the "leaky" character of the root, although they may influence the ability of a pathogenic fungus to "find" a susceptible host.

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I wish to thank Dr. C. G. Hansford and Dr. N. T. Flentje, under whose supervision this work was carried out; Dr. J. H. Warcup for continued interest and helpful suggestions; Miss J. Thomson and Mrs. T. Wickman for technical assistance; and Mr. K. Philips for the photography.

V. REFERENCES

BLAIR, I. D. (1943).—Behaviour of the fungus *Rhizoctonia solani* Kühn in the soil. *Ann. Appl. Biol.* **30**: 118.

BRAUN, H. (1930).—"Der Wurzeltöter der Kartoffel *Rhizoctonia solani K.*" Monographie zum Pflanzenschutz, No. 5, 136 pp. (J. Springer: Berlin.) (Abstr. *Rev. Appl. Mycol.* **9**: 739, (1930)). Original paper not seen.

BROWN, R. (1946).—Biological stimulation in germination. *Nature* **157**: 64.

BROWN, W. (1922).—Studies in the physiology of parasitism. IX. The effect on the germination of fungal spores of volatile substances arising from plant tissues. *Ann. Bot. Lond.* **36**: 285.

BURGES, A. (1939).—Soil fungi and root infection. *Broteria* **8**: No. 35, fasc. 2.

DOBBS, C. G., and HINSON, W. H. (1953).—A widespread fungistasis in soils. *Nature* **172**: 197.

GARRETT, S. D. (1951).—Ecological groups of soil fungi: a survey of substrate relationships. *New Phytol.* **50**: 149.

GÄUMANN, E. (1950).—"Principles of Plant Infection." (Ed. William B. Brierley.) (Crosby Lockwood & Son Ltd.: London.)

GLYNNE, M. D., and RITCHIE, W. M. (1943).—Sharp eyespot of wheat caused by *Corticium (Rhizoctonia) solani*. *Nature* **152**: 161.

KERNKAMP, M. F., DE ZEEUW, D. J., CHEN, S. M., ORTEGA, B. C., TSIANG, C. T., and KHAN, A. M. (1952).—Investigations on physiologic specialisation and parasitism of *Rhizoctonia solani*. Tech. Bull. Minn. Agric. Exp. Sta. No. 200, 36 pp.

LEBEAU, J. B., and DICKSON, J. G. (1953).—Preliminary report on production of hydrogen cyanide by a snow-mold pathogen. *Phytopathology* **43**: 581.

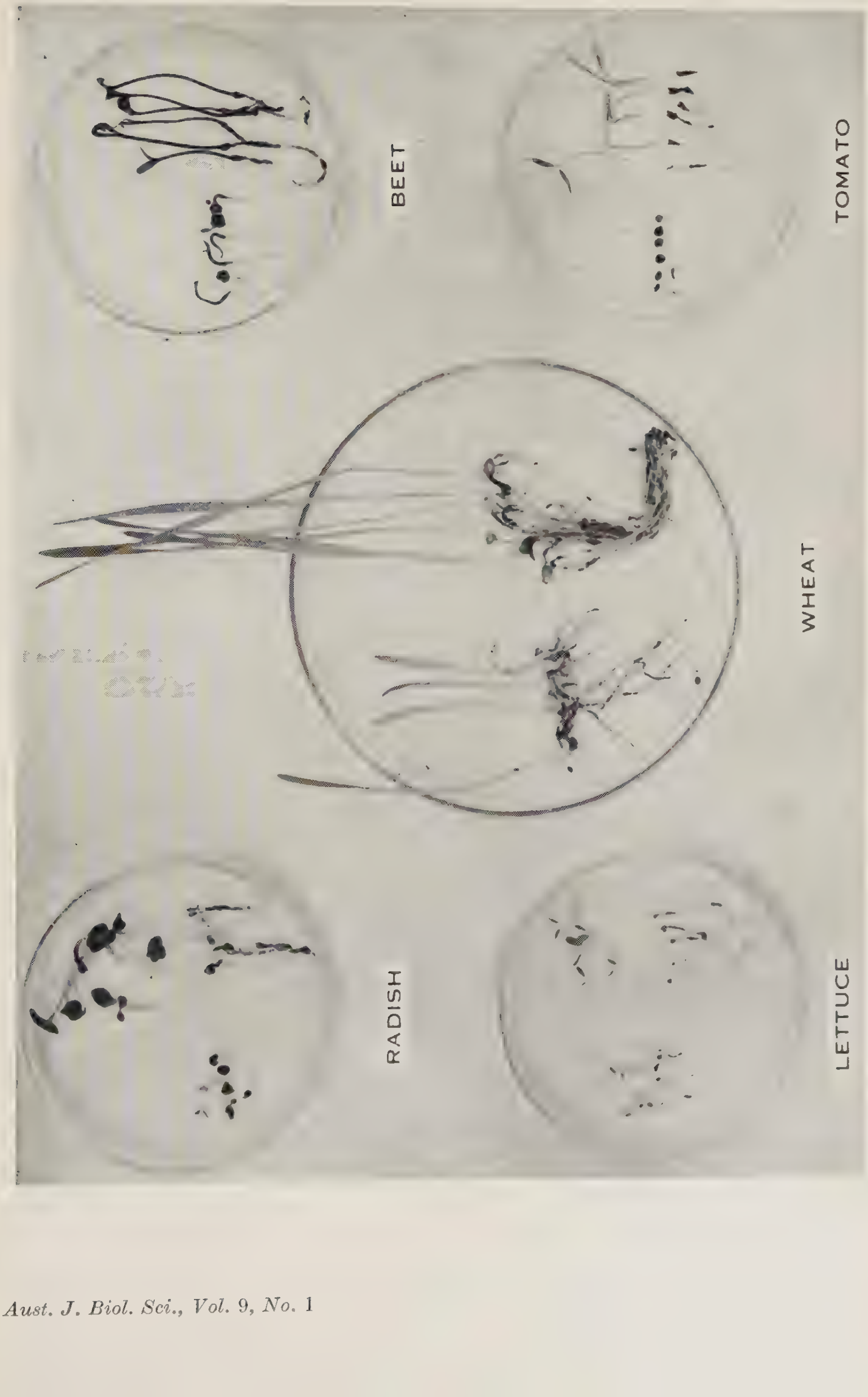
MONTEITH, J., and DAHL, A. S. (1932).—Turf diseases and their control. Bull. Green Sect. U.S. Golf Ass. No. 12, p. 85.

MÜLLER, K. O. (1924).—Untersuchungen zur Entwicklungsgeschichte und Biologie von *Hypochnus solani* P.u.D. (*Rhizoctonia solani* K.). *Arb. biol. Abt. (Anst-Reichsanst.), Berl.* **13**: 197.

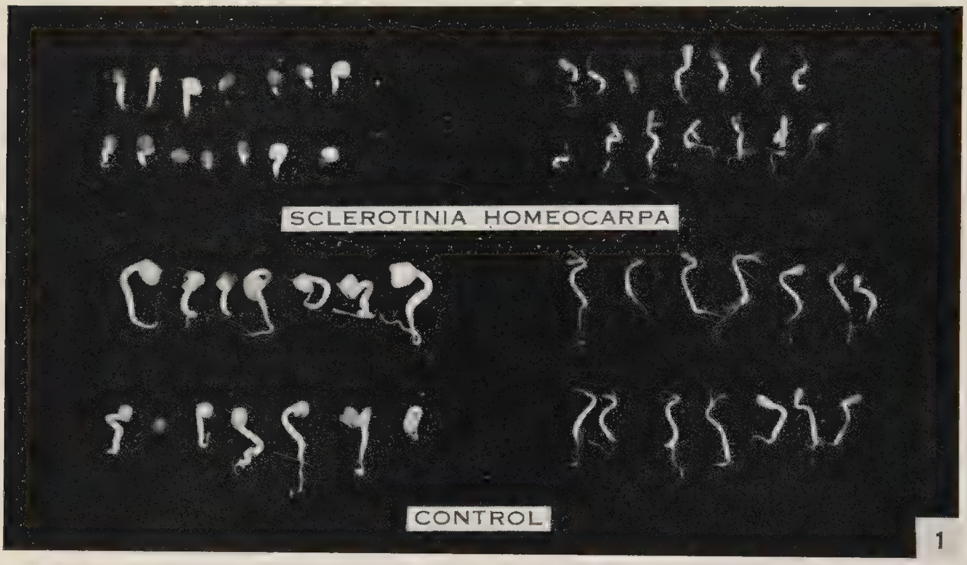
NEWTON, W., and MAYERS, N. (1935).—The physiology of *Rhizoctonia solani* Kühn. III. The susceptibility of different plants as determined by seedling infection. *Sci. Agric.* **15**: 393.

SIU, R. G. H. (1951).—"Microbial Decomposition of Cellulose, with Special Reference to Cotton Textiles." (Reinhold Publ. Corp.: New York.)

STOREY, I. F. (1941).—A comparative study of strains of *Rhizoctonia solani* (Kuhn) with special reference to their parasitism. *Ann. Appl. Biol.* **28**: 219.



The pathogenic effects of *Sclerotinia homeocarpa* on five hosts. Left, seedlings from inoculated soil. Right, seedlings from uninoculated soil.



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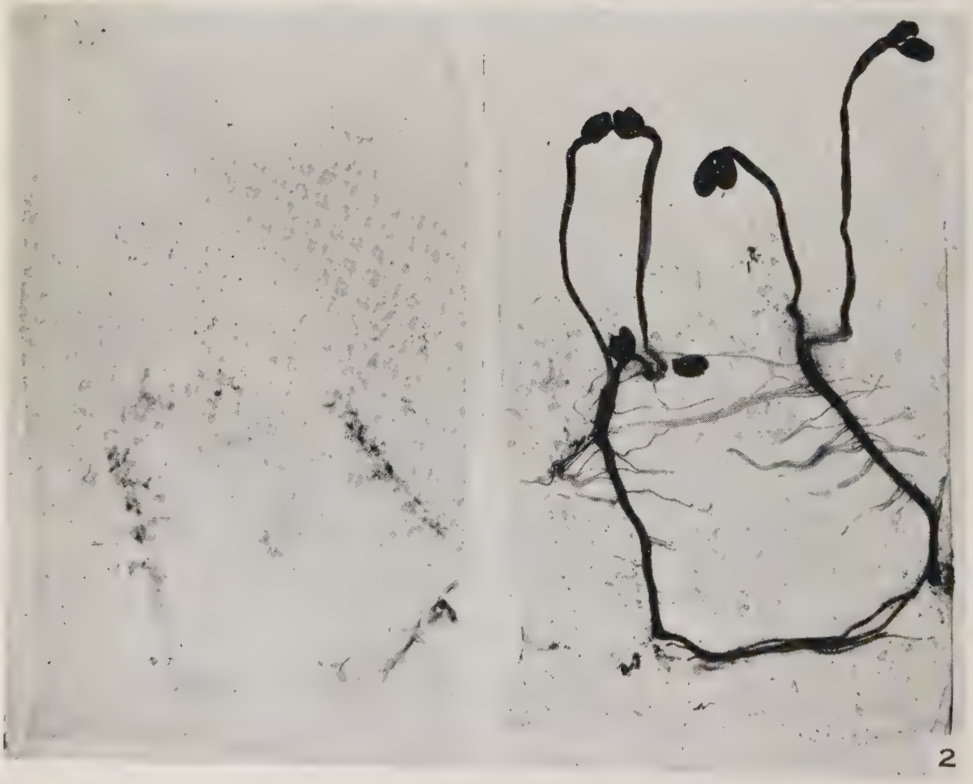

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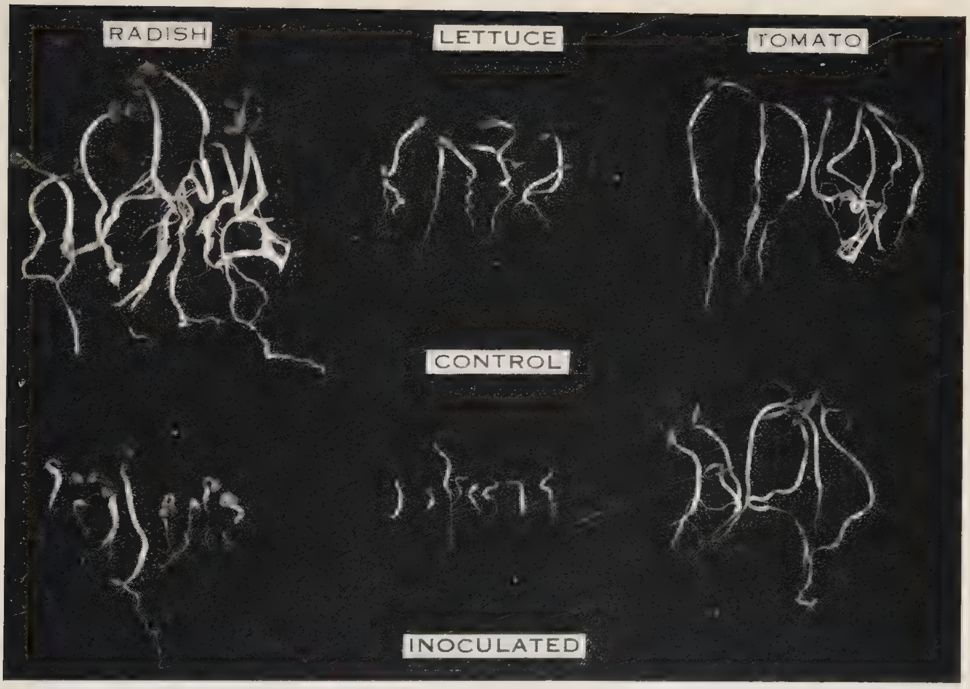
Fig. 1.—The growth of radish (left) and lettuce (right) seedlings inside "Cellophane" bags buried in uninoculated soil, and in soil inoculated with *Sclerotinia homeocarpa*.

Fig. 2.—The aggregation of *Pellicularia filamentosa* hyphae on the outside of a "Cellophane" bag directly opposite the roots of radish seedlings.



The growth of radish, lettuce, and tomato seedlings inside "Cellophane" bags buried in uninoculated soil, and in soil inoculated with *Pellicularia filamentosa*.

THE ELECTRIC PROPERTIES OF PLANT CELL MEMBRANES

I. THE ELECTRIC CAPACITANCE OF SUSPENSIONS OF MITOCHONDRIA, CHLOROPLASTS, AND CHLORELLA SP.

By A. B. Hope*

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Summary

The electric capacitance of the membranes of isolated mitochondria and chloroplasts and of *Chlorella* cells has been calculated from measurements on suspensions at frequencies from 1 kc/s to 4 Mc/s in an alternating current bridge of special design.

The capacitance per unit area is of the order of 1 μF/cm^2, similar to that of numerous animal cell membranes (nerve, red blood cell, etc.). The phase angle of the impedance representing the membrane dielectric is constant over a wide frequency range, for a given suspension, but the capacitance and dielectric loss both vary with frequency. The phase angle is less (the dielectric more "lossy") for isolated cell particles than for intact cells. Dielectric constants were calculated using measured specific capacitance and membrane thicknesses obtained from electron micrographs of chloroplasts and mitochondria. At a frequency of 1 kc/s the dielectric constant was 13 (chloroplasts) and 54 (mitochondria). The significance of the phase angle obtained in these experiments is discussed relative to Cole's (1949) suggestion that the phase angle may be related to the degree of dipole interaction in the membrane dielectric.

I. INTRODUCTION

Since the classical work on the electric resistance and capacitance of cells and cell suspensions was carried out by Fricke (1924, 1925) and later by Cole and his collaborators (see reviews by Cole 1940, 1942, 1949), little has been done to interpret membrane structure in terms of the dielectric properties. Comparatively little has been done to compare plant cell membrane properties with those of the nerve, red blood cell, marine egg, etc., which the earlier experimenters used.

Curtis and Cole (1937) examined the transverse alternating current characteristics of the *Nitella* membrane but obtained inconclusive results for the internal (sap) resistivity, suggesting that a further analysis of this cell as an electrical system is necessary. Umrath (1942) reinterpreted these results in the light of a modified theory which needs careful examination. Further, the recent results of Bennett and Rideal (1954) differ in the capacitance values, measured directly across the membrane(s), by more than an order of magnitude from those obtained by Curtis and Cole (1937) with external electrodes. These aspects are the subject of further experiments on the electric properties of *Chara*, currently proceeding.

Other work in this field of A.C. measurements on plant cells includes that of Remington (1928-9) on slices of beet tissue, which is unsatisfactory in that the current

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paths through intercellular spaces and cell walls were insufficiently defined. Iwamura (1952) showed that the electric impedance of a plasmodium of *Physarum* can be described electrically in the same terms as used with the animal experiments.

The present programme of work was designed to investigate the electrical properties of some plant cell membranes which had become of interest in other investigations (Mercer *et al.* 1955; Robertson *et al.* 1955) and eventually to try to interpret the molecular architecture of these membranes in terms of dielectric characteristics together with other known physical properties.

II. Material, Apparatus, and Experimental Method

(*a*) *Mitochondria*

Mitochondria from *Beta vulgaris* were prepared by a differential centrifugation of cell-free homogenates as described in Robertson *et al.* (1955). The final medium was 15 per cent. sucrose with KCl added to give the required conductivity. The suspension usually contained 15–30 per cent. by volume of mitochondria. A less concentrated suspension reduced the resistance and capacitance difference between suspension and medium, while more concentrated ones gave trouble with trapped air bubbles.

The time between homogenization and the electrical measurements was usually about 70 min, during which time the particles were kept at 0°C. For information about the metabolic activity of similar preparations the reader is referred to Robertson *et al.* (1955).

After measurements, the suspension was placed in narrow-bore "Pyrex" tubes and spun in the super-speed head for 10 min at 24,000 *g* to give a clear supernatant for further measurements.

(*b*) *Chloroplasts*

Chloroplasts from *Chara australis* (var. *nobilis*) were obtained by cutting and grinding clean sorted cells in glucose, straining through muslin to remove cell wall pieces, and centrifuging to bring down the chloroplasts. Starch-free cells were chosen wherever possible. The final medium was 0·5M glucose and KCl. In this tonicity the chloroplasts appeared as approximately spherical under the microscope. The time between grinding and electrical measurements was approximately 20 min.

(*c*) Chlorella

Chlorella (*pyrenoidosa*?) cells were cultured in a medium described by Emerson and Lewis (1939). After about 5 days the cells were concentrated by centrifugation, resuspended in 0·1M glucose, spun down, and finally resuspended in 0·1M glucose and KCl. The diameters of 50 or more cells were measured at a magnification of about 600× for use in calculating the specific capacitance of the cell surface.

(*d*) *Method*

The resistance and capacitance of the suspensions were measured at a number of frequencies by balancing the unknown against a substandard parallel resistance and capacitance. The former consisted of an electrolytic resistor substantially as described by Cole and Curtis (1937) which used the separation between one fixed platinized platinum electrode and another mounted on a micrometer spindle, with KCl solution between them, as the means of varying the resistance. This resistor was calibrated occasionally for resistance and capacitance against electrode separation, using temperature-stable "Welwyn" carbon resistors and air or mica capacitors. The substandard capacitors were of two ranges, depending on the experiment. One was variable from about 11 to 1,111 $\mu\mu$F with an accuracy of $\pm 0{\cdot}1$ $\mu\mu$F*, and the other from 12 to 11,112 $\mu\mu$F with an accuracy of $\pm$ 0·2 $\mu\mu$F*.

The measuring bridge is shown in the circuit diagram (Fig. 1). By careful design of the transformer *T*†, the secondary outputs from 1 and 2 have been made equal in magnitude and phase to within 0·1 per cent. over the frequency range 100 c/s to 2 Mc/s., with negligible distortion as long as the secondary voltage did not exceed 0·1 V at the lowest frequency. The error in using the transformer at 4 Mc/s was small and could be allowed for in the electrolytic resistor calibration. For frequencies up to 200 kc/s, sinusoidal voltage was obtained from a "Techtron" oscillator with a 20 : 1 transformer to match into the transformer *T*. For frequencies above this, a small spot-frequency oscillator was used, giving several volts at 500 kc/s, and at 1, 2, and 4 Mc/s. A cathode-ray oscillograph with auxiliary amplifiers was used as the null-point indicator.

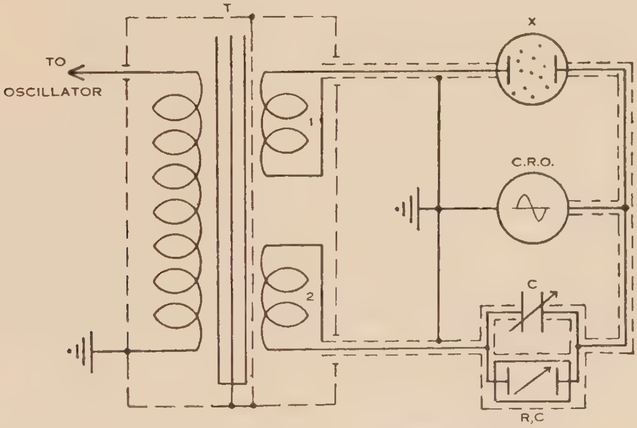

Fig. 1.—The circuit of the A.C. bridge used to measure the equivalent parallel resistance and capacitance of suspensions. The dotted lines represent electrostatic shielding. The unknown *x* was balanced by the resistance and capacitance of the electrolytic resistor *R*, *C*, and mica capacitors *C*. C.R.O. = cathode-ray oscillograph; auxiliary amplifiers not shown.

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* These were standardized with the help of the Division of Electrotechnology, National Standards Laboratories, C.S.I.R.O.

† Designed by Mr. L. Medina, Division of Electrotechnology, National Standards Laboratories, C.S.I.R.O.

(*e*) *Measuring Cell*

The cells or cell particles were suspended for measurement in a small conductivity pipette (cell constant k = resistance/resistivity $\simeq$ 0·5) in which 0·2 ml of suspension were required to cover the electrodes. The contents were kept mixed and suspended by occasionally returning the liquid to a small glass reservoir on the end of the pipette using pressure on a rubber bulb on the other end. No effect of O_2 supply on resistance or capacitance was evident, but O_2 may never have been limiting owing to the mixing which was necessary to keep the particles in random suspension.

Mitochondria in similar suspensions at 25°C have an appreciable O_2 uptake, though less than that with added substrate (see Robertson *et al.* 1955). No evidence about the level of metabolic activity of isolated chloroplasts or of *Chlorella* cells has been obtained.

Both the electrolytic resistor and measuring pipette were kept at constant temperature (usually $25\pm0{\cdot}02$°C) in a water-bath.

III. THEORY

Although the theory of this type of measurement has been developed and discussed by Fricke (1924, 1925) and Cole (1928), some of the main concepts will be repeated.

(i) The mean resistivity (r_2) in Ωcm of homogeneous spheres at low frequency in random suspension is given in terms of the resistivity of the suspension (r) and the medium (r_1) and the volume concentration ρ of the spheres, as:

$$\frac{1-r_1/r}{2+r_1/r}=\rho\left(\frac{1-r_1/r_2}{2+r_1/r_2}\right) \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

(Maxwell 1873).

If the spheres have a relatively non-conducting exterior surrounding a homogeneous interior of much lower resistivity, and the systems dealt with in these experiments are thought to be of this sort, then the non-conducting volume concentration ρ_0 is given approximately by

$$\rho_0=2\left(\frac{1-r_1/r}{2+r_1/r}\right), \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

where equation (**2**) is obtained from equation (**1**) by putting $r_2=\infty$.

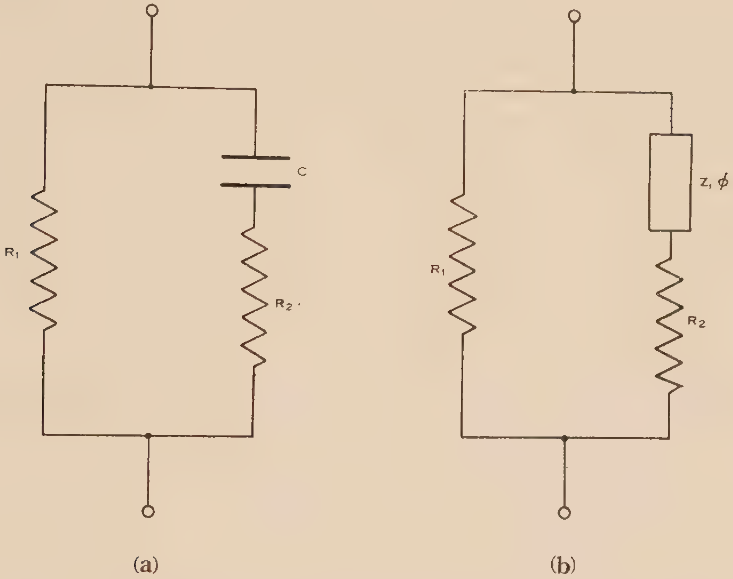
As Cole (1940) has pointed out, ρ in equation (**1**) would need to be determined to within 2 per cent. to reveal any difference in membrane resistivity between ∞ and 25 Ωcm^2 in his suspensions of Hipponoë eggs.

In the present series of experiments, where the volume concentration could be determined independently (using a haemocytometer), it was approximately equal to the non-conducting volume concentration ρ_0 as calculated from equation (2). In the absence of an accurate method of measuring ρ, we assume the suspended particles have an unknown but high resistance at low frequencies.

(ii) The capacitance per unit area is calculated from the difference in capacitance between suspension and medium, at each frequency, with correction made for the capacitance of the electrolytic resistor. Then at low frequencies, the capacitance c_2 in F/cm² of the surface of the suspended spheres is given (Cole and Cole 1936*a*) as:

$$c_2=\frac{2C_pk}{(2+r_1/r)(1-r_1/r)a}, \quad \ldots\ldots\ldots\ldots\ldots\ldots(3)$$

where C_p is the capacitance of the suspended particles in farads, k the cell constant, a the radius of the spheres in cm, and r_1 and r are as before.



(a) (b)

Fig. 2.—Equivalent circuits representing the electrical properties of suspensions. (*a*) The membrane is represented by a pure capacitance C, resistance to current flow through the medium by R_1, and the resistance of the interior of the suspended particle by R_2; (*b*) the membrane is represented by an impedance Z with a phase angle ϕ constant with frequency.

(iii) The resistance and capacitance data are conveniently represented by plotting for the various frequencies the series resistance R_s against series reactance X_s, both in ohms, where

$$X_s=1/\omega C_s$$

($\omega=2\pi\times$frequency, and C_s is the series capacitance). As the measuring bridge gives equivalent parallel values R_p and C_p, the series coordinates are calculated from the usual transformations:

$$R_s=\frac{R_p}{1+(\omega R_pC_p)^2};\; X_s=\frac{\omega R_p{}^2C_p}{1+(\omega R_pC_p)^2}. \quad \ldots\ldots\ldots\ldots(4)$$

The equivalent circuit, which is a formal representation of the electric properties of the suspension, reduced to a two-terminal network, can be shown as in Figures 2 (*a*) and 2 (*b*). C or Z represent the membrane capacitance or impedance respectively. R_1 represents the resistance to current flow in the medium between the spheres and

R_2 that due to electrolyte inside the membrane. At low frequencies when no current passes through the spheres the parallel measured resistance is R_1 while at very high frequencies the reactance of C or the impedance of Z has a low value, and current flows through R_2. The resultant resistance is R_1 and R_2 in parallel. When the membrane has a pure capacity (Fig. 2(*a*)) and the R_s at each frequency is plotted against the corresponding X_s, the result is a semi-circle with the centre on the R_s-axis and cutting this axis at R_1 $(\omega = 0)$, and $\frac{R_1R_2}{R_1+R_2}$ $(\omega = \infty)$. When, however, the membrane has a variable impedance Z with a constant phase angle (Fig. 2 (*b*)), Cole (1928) showed that the impedance locus is an arc of a circle with the centre below the R_s-axis. The phase angle of the impedance is then half the angle subtended at the centre of the circle by the arc. The measured capacitance and equivalent resistance representing the dielectric loss in this case are both functions of the frequency.

Table 1

PARALLEL RESISTANCE AND CAPACITANCE AGAINST FREQUENCY FOR A SUSPENSION OF BEET MITOCHONDRIA

f (kc/s)	1	2	5	10	20	50	100	200	500	1000	2000
R_p (Ω)	3741	3723	3705	3683	3656	3606	3542	3445	3287	3139	3012
C_p ($\mu\mu$F)	15	49	52	45	35	25·1	18·8	12·4	5·9	2·7	0·8

IV. Results

(*a*) *Mitochondria*

The low-frequency resistance of a suspension of beet mitochondria was found to decrease continually at 25°C due to leakage of electrolyte from the interior to the medium, either because of gradual breakdown of the membranes of the whole population, or sudden complete breakdown of a certain number per unit time. For this reason the first definite results were got at 0°C with the suspension in a stirred ice-water mixture where the resistance drift was quite small.

Table 1 gives a typical set of R_p, C_p with frequency. The capacitance difference C_p between suspension and medium rises with increasing frequency and then falls, while the resistance falls continually. The decrease in C_p with increasing frequency is partly due to actual membrane capacitance decrease and partly due to the fact that the parallel capacitance depends on frequency even when C in Figure 2 (*a*) is constant, i.e.

$$C_p = \frac{C}{1+(\omega R_2 C)^2}. \quad \dots\dots\dots\dots\dots\dots\dots (5)$$

The frequencies at which C_p become appreciably less than C depend on R_2, the element in Figure 2 representing the internal resistance.

Figure 3 shows the plot of $X_s : R_s$, these being calculated from Table 1. The impedance locus is an arc of a circle with the centre well below the R_s-axis, corresponding to the presence of a variable impedance element (cf. Fig. 2 and Section III).

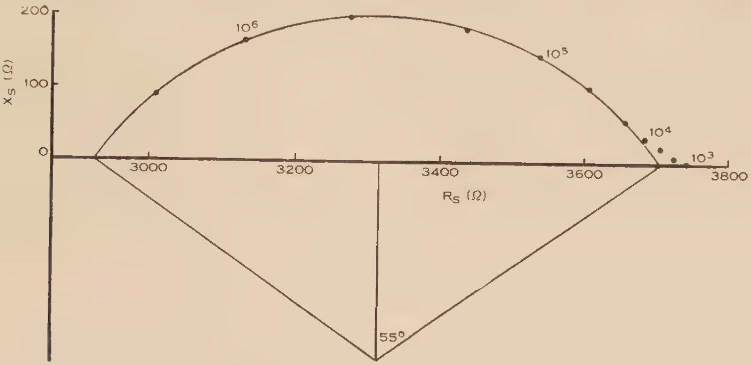


Fig. 3.—Impedance locus for a suspension of beet mitochondria. R_s = equivalent series resistance; X_s = series reactance, calculated from the measured parallel values shown in Table 1. The frequencies are in c/s.

The phase angle ϕ is constant over most of the frequencies and equals 55°. The flattening of the locus at the low-frequency end probably represents polarization of the membrane. In Figure 4 (a), $\log_{10}C_p$ is plotted against $\log_{10}f$, f being the frequency in c/s. Over a portion of the middle-frequency range the relation is linear. At the lowest frequencies the capacitance tends to decrease with decreasing frequency and in some experiments negative capacitances were found, corresponding to an inductive reactance being present in the system. In other experiments (e.g. curve (b), Fig. 4)

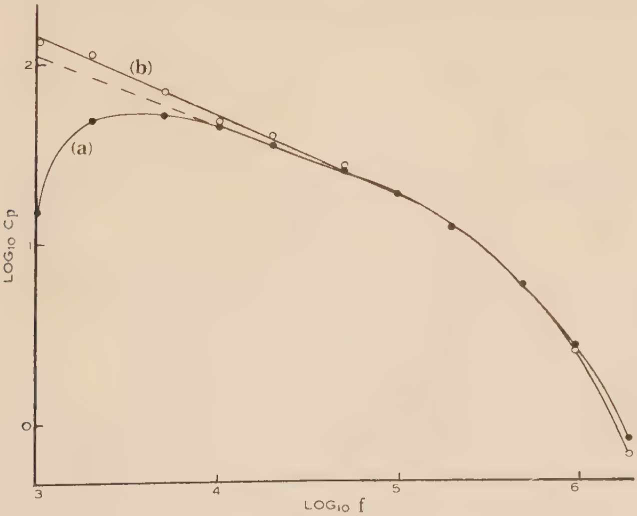


Fig. 4.—$\text{Log}_{10}C_p$ plotted against $\log_{10}f$. (a) For the same suspension as Figure 3 and Table 1, and (b) for a similar suspension.

the relation between $\log_{10}C_p$ and $\log_{10}f$ did not depart from linearity at the lowest frequency used. Differences between preparations are not yet explained.

At an arbitrary frequency of 1 kc/s the mean membrane capacitance in eight experiments was calculated, using equation (**3**), as $2{\cdot}8 \pm 1{\cdot}2$ μF/cm^2. Since the effect of the membrane is negligible at infinite frequency, the extrapolation to $\omega = \infty$ enables r_2, the mean resistivity of the interiors of the mitochondria, to be calculated using equation (**1**). This was on the average three times that of the medium at 0°C (10 experiments) and one and a half times the medium resistivity at 25°C (six experiments). r_2 might be expected to be less than r_1 since, as shown by Robertson *et al.* (1955), the concentration of K^+, Na^+, and Cl^- is higher in the mitochondria than in the medium for similar external concentrations. The implications of this will be discussed in Section V.

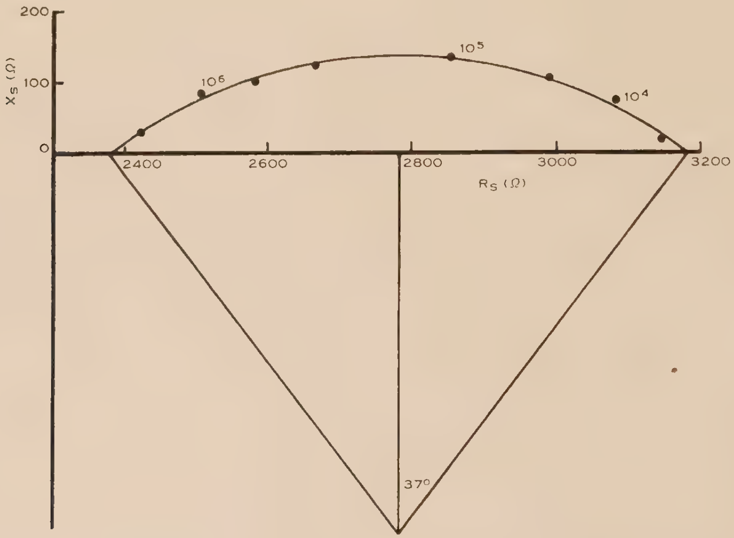


Fig. 5.—Impedance locus (X_s : R_s) for a suspension of *Chara* chloroplasts. Frequencies in c/s.

For mitochondrial suspensions measured first at 0°C and then 25°C, the impedance loci were similar to that in Figure 3, but sometimes with a smaller phase angle at 25°C. However, this difference is probably not significant as R_p was falling with time, and the readings were taken from low to high frequency which would tend to flatten out the locus.

(*b*) *Chloroplasts*

Figure 5 shows a typical impedance locus for a suspension of chloroplasts in 0·5M glucose and KCl at 25°C. The phase angle is less than that of the mitochondria, being 37°. The infinite-frequency resistance again leads to $r_2 > r_1$ in all experiments. The mean specific capacitance at 1 kc/s was 1·6 μF/cm^2 in four experiments.

(c) Chlorella

The impedance loci at 0 and 25°C for a suspension of *Chlorella* cells in 0·1M glucose and KCl is shown in Figures 6 (*a*) and 6 (*b*). The phase angles are equal to 69° and 67° respectively. The series reactance rises again at the highest frequencies indicative of an inner membrane surrounding a more conducting region, possibly the chloroplast or nucleus. This effect is similar to that obtained by Cole and Cole (1936*a*, 1936*b*) with *Asterias* and *Arbacia* eggs and attributed to the nucleus. Because of this second impedance element in the system the extrapolation to infinite frequency carries less conviction. However, this extrapolation gave a relatively constant r_2 of 980 ± 200 Ωcm at 25°C (six experiments) independent of the medium concentration.

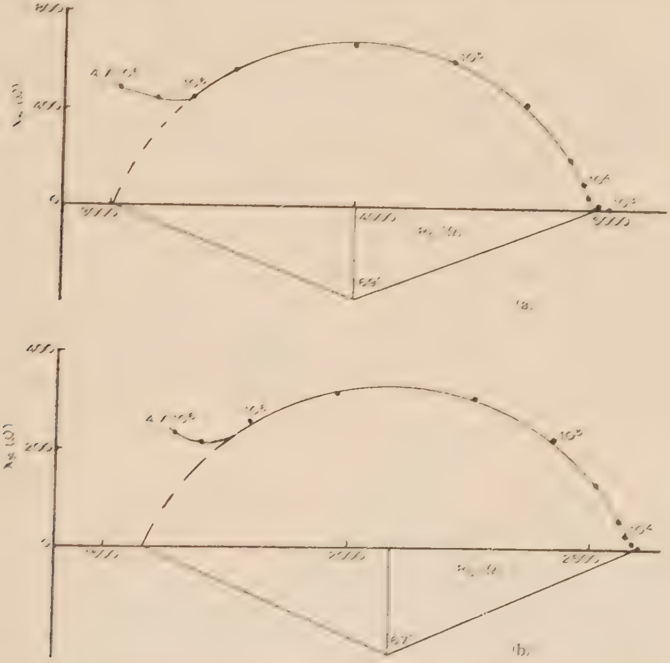


Fig. 6.—Impedance loci for a suspension of *Chlorella* cells: (*a*) at 0°C, and (*b*) at 25°C. Frequencies in c/s.

The mean capacitance/unit area of the main impedance element at 1 kc/s was 1·0 ± 0·4 μF/cm² (six experiments), while the mean phase angle was 71 ± 8°. These values are comparable with those quoted in Cole (1942) from his unpublished results, viz. $c_2 = 0{\cdot}33\ \mu\text{F/cm}^2$, $r_2 = 460\ \Omega\text{cm}$, $\phi = 80°$.

V. Discussion

(a) *The Physical System*

Electron microscope and other studies (Mercer *et al.* 1955; Farrant *et al.*, unpublished data 1955) show that the chloroplast of *Nitella* and the beet mitochondrion are bounded at their surfaces by thin membranes. That of the chloroplast is 70 Å or more thick, while that of the isolated mitochondrion is *c.* 170 Å thick.

In the *Chlorella* cell, published electron micrographs (Albertson and Leyon 1954) do not show any membrane between the cell wall and cytoplasm nor around the chloroplast. However, Hodge and Hope (unpublished data 1954) by staining with phosphotungstic acid after osmium fixation, demonstrated a membrane surrounding the chloroplast of *Chlorella* but not a cytoplasmic or vacuolar membrane with certainty. The electrical measurements just described indicate that a membrane with a large capacitance surrounds a large proportion of the cell contents, since the non-conducting volume concentration was approximately equal to the total volume concentration measured by counting cells and measuring their diameters in a haemocytometer. The mean diameter of the cells was usually c. 5 μ.

The interior of the isolated beet mitochondrion in section does not show much organized structure of the sort found by Palade (1953) in animal mitochondria but this may be due to limitations of fixation technique. From chemical analysis the beet mitochondria contain large amounts of protein. These mitochondria, which are approximately spherical, have a mean diameter of approximately 1 μ.

The chloroplasts in 0·5M glucose, judging from the appearance of electron micrographs of sections of *Nitella* and of isolated swollen chloroplasts, probably have a slightly disorganized interior with large spaces between the groups of lamellae, due to swelling from an oblate spheriod to a sphere of diameter 8-9 μ. We can conclude that at least for the mitochondrion and chloroplast the equivalent circuit of Figure 2 is a reasonable representation of the electrical elements in the suspensions. At least one further capacitative reactance should be added for *Chlorella* but this is not important until high frequencies (>1 Mc/s) are reached.

(*b*) *The Impedance Locus*

It has been seen that in the suspensions measured, in every case when series resistance is plotted against series reactance, the points fall accurately on the arc of a circle with the centre below the R_s-axis over a considerable range of frequency. Deviations at either end of the frequency range are attributed to polarization (Figs. 3 and 6), inductive behaviour of the membrane (Fig. 4 (*a*)), or to enclosed reactances (Fig. 6). From the considerations in Section III, the circular arc locus means that the system contains a variable impedance element with the property that as the frequency changes both the capacitative and resistive (dielectric loss) components change, but the phase angle remains constant in the frequency range considered. If c' is the real or conservative part of the capacitance it can be shown that

$$c' = \frac{\bar{c}\sin(\alpha\pi/2)}{\omega^{1-\alpha}}, \quad \dots\dots\dots\dots\dots\dots\dots\dots (6)$$

where $\bar{c}$ and α are constants. α is related to the phase angle: $\phi = \alpha\pi/2$. Thus if $\alpha = 0$, $c' = 0$; if $\alpha = 1$, $c' =$ constant; while if $\alpha = 0{\cdot}5$ (approximately the chloroplast behaviour), $c' = 0{\cdot}707\bar{c}/\sqrt{\omega}$. α is termed by Cole the "loss constant". Figure 4 shows that the variation of capacitance with frequency is consistent with equation (**6**) and the slope of the linear part enables us to calculate α and ϕ as a check on the ϕ value obtained from Figure 3. From Figure 4, curve (*a*), $\alpha = 0{\cdot}62$ and therefore $\phi = 56°$ (55° in Fig. 3). α calculated for all the experiments varied from 0·41 to 0·89 ($\phi = 37$-$80°$).

Its significance in terms of the molecular structure of the dielectric is not yet clear, although it has been found that α measured for a solution of β-lactoglobulin increased as concentration increased (Shaw, Jansen, and Lineweaver 1944). Also lowering the temperature of ice (Murphy 1934) and glycerin (Morgan 1934) increased α. Thus Cole (1949) suggests by analogy that unit loss constant means the complete replacement of viscous opposition to dipole rotation by intermolecular forces, which are negligible in dilute solutions. However, the data for ice are inadequate at the highest frequencies and other workers find $\alpha = 0$ at various temperatures (Auty and Cole 1952).

If the above suggestion is accepted tentatively, the membranes dealt with here may be thought of as lying somewhere between the completely "frozen" arrangement of molecules ($\alpha = 1$, e.g. the *Arbacia* egg) and an open aggregation with no intermolecular forces ($\alpha = 0$, a dilute "solution" of dipoles). It would be interesting to see if α (and ϕ) varied with the degree of stretching of the particle membrane during swelling (the chloroplast would be a suitable material). Interpretations of the loss constant in terms of a single relaxation time ($\alpha = 0$) of the molecules following the electric field or a distribution over a large range of times ($\alpha > 0$) offers no more in the way of an explanation in physical terms (Cole and Cole 1941).

TABLE 2

SPECIFIC CAPACITANCE OF THE MEMBRANES AT 1 KC/S, MEMBRANE THICKNESS, AND CALCULATED DIELECTRIC CONSTANT

Membranes of	Specific Capacitance c_2 (μF/cm^2)	Thickness t (Å)	Dielectric Constant ϵ (at 1 kc/s)
Mitochondria	2·8	170	54
Chloroplasts	1·6	70	13
Chlorella	1·0	?	?

It is perhaps significant that for the cell particles in the absence of their normal environment α is less than for intact cells (*Chlorella*) and tissue (potato—Cole 1932; beet—Hope, unpublished data). Further discussion of this will be fruitful only when a sound theoretical basis is available.

(c) *The Specific Capacitance and Dielectric Constant*

The specific capacitance of the membranes studied, calculated for a frequency of 1 kc/s, together with the dielectric constant at this frequency are given in Table 2. The latter has been calculated as for a parallel plate capacitor assuming macroscopic behaviour and

$$c_2 = \epsilon_f/(4\pi t \times 9 \times 10^{11}), \qquad \ldots\ldots\ldots\ldots\ldots\ldots (7)$$

where ϵ_f is the dielectric constant at frequency f, and t the membrane thickness. c_2 is in F/cm^2 and t is in cm.

The differences in thickness and dielectric constant do not give us any confidence in supposing that plant membranes have a common structure, even allowing for uncertainties in the values of t. The zero-frequency ("static") dielectric constant is obviously larger than at 1 kc/s. Equation (**6**) suggests a value of ∞ so this equation cannot describe the membrane behaviour outside the frequency range studied. Unfortunately direct measurement of c_2 below the frequencies used here becomes very difficult and the inductive behaviour becomes prominent. Further information is needed on the change of dielectric constant with frequency as a step towards estimation of the dipole moment to clarify our ideas on the membrane structure. At low frequencies some suspensions of mitochondria and *Chlorella* were balanced by a negative capacitance, indicating an inductive reactance. This has been discussed by Cole (1947) and Teorell (1951), who attributed it to non-linearity (rectification) in the membrane.

(*d*) *The Internal Resistivity*

The constancy of the internal resistivity of the *Chlorella* cells in these investigations (980 Ωcm, independent of the medium) must mean that equilibrium with the external medium is not established in the time of the experiment. No large vacuoles are evident in the electron micrographs of sections of this cell so this resistivity is presumably that of the cytoplasm and possibly of the chloroplast as well.

On the other hand, in the mitochondrial suspensions, the evidence suggests an internal resistivity one and a half to three times that of the medium over a range of concentrations of the latter. Thus adjustment has probably been made to the new environment in which KCl and sucrose replaced sucrose. This is consistent with the results of Robertson *et al.* (1955) who found adjustment to be 50 per cent. complete in *c.* 6 min. However, in those experiments the internal concentrations of K^+, Na^+, and Cl^- were in excess of those of the medium at low concentrations. The lower conductivity of the interior in the present experiments must therefore be due to a lower mobility of ions inside the mitochondria. Thus, since the conductivity is a function of the product of concentration and ion conductance, it can be seen that the latter is less inside the mitochondria than in aqueous solution by a factor of 3 at 0°C and 1·5 at 25°C. This could be brought about by the binding of some of the ions in regions low in water and high in protein or lipo-protein concentration. The temperature coefficient of the resistivity (twice that of the medium) could also be consistent with this. Some effect similar to this has been demonstrated by Robertson and Honda (unpublished data) in their investigation of the ionic relations of beet mitochondria. The effect of a high concentration of colloid on ion conductance through increased viscosity only is probably negligible (McKenzie 1948).

Electron micrographs of sections of mitochondria (Farrant *et al.*, unpublished data 1955) generally show an interior in which OsO_4 has been strongly reduced to Os, indicating a high concentration of protein, etc., relative to the rest of the cytoplasm.

Similarly, in the chloroplast, ion mobility would probably be reduced in this way although enough data have not been assembled. For example it might be expected that r_2/r_1 would be less for more swollen chloroplasts.

VI. CONCLUSIONS

The membranes of mitochondria, chloroplasts, and *Chlorella* cells behave electrically as "lossy" capacitors in which the capacitance and equivalent resistance due to losses both change with frequency but the phase angles of which remain constant over a wide frequency range. The phase angle is possibly related to the measure of molecular interaction between dipoles in the membrane, but confirmation of this theory is still absent.

The capacitances are similar to those found in various animal cell membranes and for an artificial bimolecular film of lecithin and tanned egg albumin (Dean, Curtis, and Cole 1940), but the phase angles of the membranes of extracted cell particles are less than those of inact cells.

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VIII. REFERENCES

ALBERTSON, P. Å., and LEYON, H. (1954).—*Exp. Cell Res.* **7**: 288.
AUTY, R. P., and COLE, R. H. (1952).—*J. Chem. Phys.* **20**: 1309.
BENNETT, M. C., and RIDEAL, E. (1954).—*Proc. Roy. Soc.* B **142**: 483.
COLE, K. S. (1928).—*J. Gen. Physiol.* **12**: 29.
COLE, K. S. (1932).—*J. Gen. Physiol.* **15**: 641.
COLE, K. S. (1940).—*Cold Spr. Harb. Sym. Quant. Biol.* **8**: 110.
COLE, K. S. (1942).—*Tabul. Biol. Hague* **19**: 24.
COLE, K. S. (1947).—Publ. Inst. Biofis. Braz. No. 1.
COLE, K. S. (1949).—*Proc. Nat. Acad. Sci., Wash.* **35**: 558.
COLE, K. S., and COLE, R. H. (1936*a*).—*J. Gen. Physiol.* **19**: 609.
COLE, K. S., and COLE, R. H. (1936*b*).—*J. Gen. Physiol.* **19**: 625.
COLE, K. S., and COLE, R. H. (1941).—*J. Chem. Phys.* **9**: 341.
COLE, K. S., and CURTIS, H. J. (1937).—*Rev. Sci. Instrum.* **8**: 333.
CURTIS, H. J., and COLE, K. S. (1937).—*J. Gen. Physiol.* **21**: 189.
DEAN, R. B., CURTIS, H. J., and COLE, K. S. (1940).—*Science* **91**: 50.
EMERSON, R., and LEWIS, C. M. (1939).—*Amer. J. Bot.* **26**: 808.

Fricke, H. (1924).—*Phys. Rev.* **24**: 575.

Fricke, H. (1925).—*Phys. Rev.* **26**: 678.

Iwamura, T. (1952).—*Cytologia, Tokyo* **17**: 322.

Maxwell, J. C. (1873).—"Treatise on Electricity and Magnetism." (Clarendon Press: Oxford.)

McKenzie, H. A. (1948).—*J. Coun. Sci. Industr. Res. Aust.* **21**: 210.

Mercer, F. V., Hodge, A. J., Hope, A. B., and McLean, J. D. (1955).—*Aust. J. Biol. Sci.* **8**: 1.

Morgan, S. O. (1934).—*Trans. Electrochem. Soc.* **65**: 109.

Murphy, E. J. (1934).—*Trans. Electrochem. Soc.* **65**: 133.

Palade, G. E. (1953).—*J. Histochem. Cytochem.* **1**: 188.

Remington, R. E. (1928–9).—*Protoplasma* **5**: 338.

Robertson, R. N., Wilkins, Marjorie J., Hope, A. B., and Nestel, Lydia (1955).—*Aust. J. Biol. Sci.* **8**: 164.

Shaw, T. M., Jansen, E. F., and Lineweaver, H. (1944).—*J. Chem. Phys.* **12**: 439.

Teorell, T. (1951).—*Z. Electrochem.* **55**: 460.

Umrath, K. (1942).—*Protoplasma* **36**: 584.

EFFECT OF NaCl IN WATER CULTURE ON THE ION UPTAKE AND GROWTH OF *ATRIPLEX HASTATA* L.

By R. F. Black*

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Summary

Atriplex hastata is taken as an example of a halophytic member of the genus. As a seedling it is shown to be tolerant to NaCl concentrations approximating that of sea-water (0·6M). Growth, however, is severely inhibited in concentrations above 0·3M. For comparable concentrations, the chloride contents of the mature leaves are lower than those of either *A. vesicaria* or tomato plants. The root systems of *A. hastata* exhibit considerably higher chloride contents on a dry matter basis than the mature leaves. The total of the mature leaf cations analysed greatly exceeded the total of the inorganic anions. This condition is shown to agree with what would be expected, assuming a simple Donnan equilibrium existed between the mature leaves and the external culture solution, where the concentration of the latter is 0·2M or above.

In the culture solutions containing Na and K in approximately equivalent concentrations, the equivalents of Na in the mature leaves is about $\frac{1}{4}$ to $\frac{1}{3}$ of the equivalents of K. Incomplete mutual competition is shown to exist between the Na and K uptakes of the mature leaves. The functional aspects of the partial substitution of K by increasing amounts of Na, and the significance of the Donnan equilibrium agreement, are discussed.

I. Introduction

The work reported in this paper is part of a general study on the autecology of Australian members of the genus *Atriplex*. It is widely known that the xerophytic indigenous species from arid and semi-arid regions accumulate large amounts of chloride in their leaves, and there is no doubt that the name "saltbush" arose from this fact. Chloride data obtained by Wood (1925) for the leaves from field specimens of a number of xerophytic *Atriplex* species, illustrate the high concentrations typical of the genus. They also show that seasonal variations are the chief cause of fluctuations in the chloride levels, the highest values being obtained after prolonged periods of drought. The best worked out species, *Atriplex vesicaria* Heward, showed a variation from 8·5 to 14·3 per cent. chloride of dried leaf material.

Preliminary experiments using plants of *A. vesicaria*, tomato, and *A. hastata*, grown in water cultures and under glass, showed that *A. hastata* took up a remarkably small amount of chloride for comparable external concentrations (Figs. 1 and 2). It was decided to examine further the salt-uptake behaviour of *A. hastata* in both leaves and roots, with particular reference to the cation-anion balance of the cell sap, and to extend this study at a later date to an indigenous perennial species such as *A. vesicaria*. In this way it is intended to make a physiological comparison within the one genus of a typical halophyte (*A. hastata*) found in coastal salt-marsh and strand communities, and a typical xerophyte (*A. vesicaria*) which is not normally found on saline soils (Osborn and Wood 1923).

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II. Methods

(*a*) *Growth Media*

Water cultures were used in all experiments as previous work with *Atriplex* (Black 1951) had shown that sand cultures were quite unsuitable for low-level chloride studies on high-chloride accumulators such as *A. vesicaria.* All solutions were prepared according to the formula for solution 1 of Hoagland and Arnon (1938), and then NaCl was added in the required amounts to give the following series: 0, 0·005, 0·01, 0·02, 0·05, 0·1, 0·2, 0·3, 0·4, 0·5, and 0·6M NaCl.

The solutions were contained in 4-l. enamelled cans with pressed-wood covers drilled with $\frac{3}{8}$ in. holes. Distilled water and A.R. nutrient salts were used throughout the experiments. A hand bubbler was used when watering (at approximately weekly intervals) to aerate the solutions and stir up any settled nutrients.

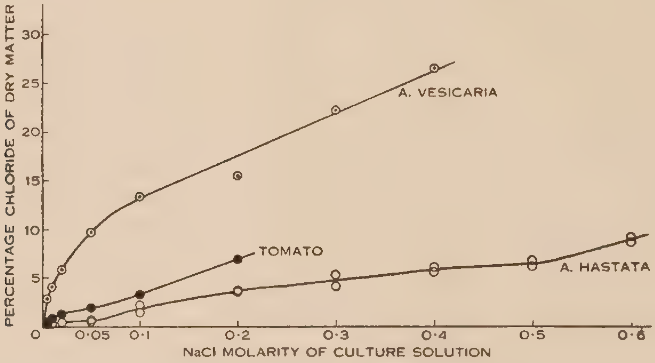

Fig. 1.—Chloride uptakes for three species expressed on basis of mature leaf dry weight, and plotted against external NaCl concentration. Chloride contents for 0 NaCl treatment: *A. vesicaria* 0·05 per cent., tomato 0·05 per cent., *A. hastata*—A < 0·01 per cent., B = 0·03 per cent.

(*b*) *Plant Culture*

The preliminary experiments using *A. vesicaria* and tomato were set out in single treatments, each can finally containing four plants. For *A. hastata*, duplicate treatments were set up, and the plants were thinned out to one per can.

The *Atriplex* seeds were germinated on moist blotting-paper, and were transplanted to the culture solutions at the cotyledon stage. "Burwood Prize" tomato seedlings were raised in a sand culture. The seedlings were initially set into low-salt cans (up to 0·05M for tomatoes, and 0·1 or 0·2M for *Atriplex*), and then those required for the high concentrations were moved up the series at 2- to 4-day intervals. In this way the salt tolerances of the seedlings were determined. These were: tomato 0·2M, *A. vesicaria** 0·4M, and *A. hastata* 0·6M.

The time allowed for growth differed according to the growth rate of the species. Actual times were: tomato 4 weeks, *A. hastata* 16 weeks, and *A. vesicaria*

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* Later experiments with a different seed batch of this species have shown that the seedlings may survive indefinitely, but with very little growth, in culture solutions containing 1M NaCl.

25 weeks. The growth in all series at or near the time of sampling is illustrated in Plate 1. With *A. hastata* plants only, flowering had commenced in most of the larger plants at the time of sampling. In this species, the dry weight figures for the roots and tops (Fig. 8) show a marked inhibition of growth for NaCl concentrations above 0·3M.

(*c*) *Sampling*

The harvesting of the three species was done in summer months. Healthy mature leaves were sampled early in the morning, their petioles removed, and the fresh weight obtained. The samples were then oven-dried at 105°C. The *A. vesicaria* samples were washed in distilled water for 1½ min before drying to remove the significant amount of surface chloride left by collapsed vesicles (Black 1954). Young growing portions of the root systems of *A. hastata* were sampled, washed in 2-3 changes of distilled water, and then oven-dried. From some of the dried *A. hastata* plants, samples were taken of terminal foliage buds including the first pair of young leaves down from the bud. Petioles were removed where possible. Samples of the nutrient solutions were retained for checking purposes. In sampling and analysis, the duplicate treatments of *A. hastata* were referred to as series A and B.

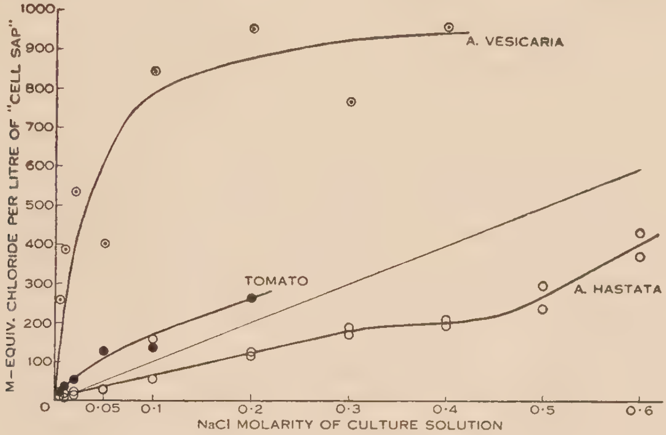

Fig. 2.—Chloride uptakes expressed as equivalents on mature leaf moisture basis, and plotted against external NaCl concentration. Chloride contents for 0 NaCl treatment: *A. vesicaria* 3, tomato 2, *A. hastata*—A < 1, B = 2.

(*d*) *Analysis*

To obtain a suitable extract from which the principal inorganic ions could be determined, the dried plant material was boiled for 1 hr in distilled water in a ratio of approximately 1 : 500, and then, after cooling, made up to a known volume at about 1 : 1000. This treatment removed more than 99 per cent. of the chloride present in the *A. vesicaria* leaf, which was the most resistant to decomposition by boiling.

Chlorides were determined in the unfiltered extracts by the electrometric method of Best (1929). Sodium, potassium, and soluble calcium levels were determined by the flame photometer after filtering. The water-soluble phosphate was estimated colorimetrically in the filtered extracts using the blue phosphomolybdate method.

Nitrates were determined on fresh samples of dried and ground mature leaf material by a modification of the 3, 4-xylenol method (Piper and Lewis, unpublished data 1951). In this method the bulk of the organic matter is eliminated by adding cupric sulphate to the extracting solvent (distilled water), the excess of copper being subsequently precipitated by the addition of calcium oxide and magnesium carbonate. The method was designed for use with soils, but can be used for plant materials where interference from certain organic constituents is not too great. Recovery checks, where nitrate was added to the leaf material before final oven drying, were about 85-95 per cent. The results were thus considered suitable for the purpose, since the nitrate present was only a small fraction of the total anion content of the leaf.

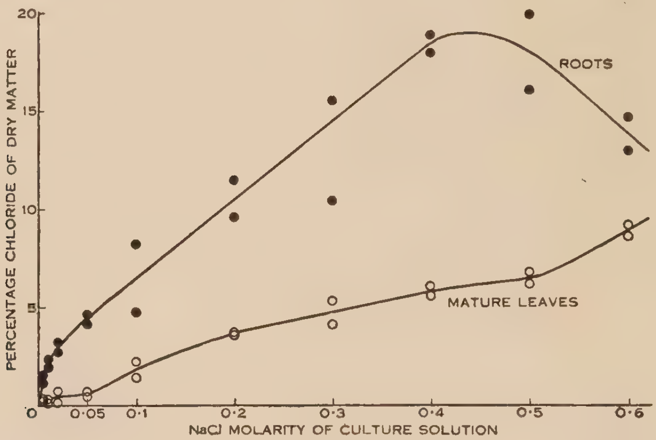

Fig. 3.—Comparative chloride uptakes on a dry matter basis of the mature leaves and roots of *A. hastata.* Chloride contents for 0 NaCl treatment: mature leaves—A < 0·01 per cent., B = 0·03 per cent.; roots—A = 0·06 per cent., B = 0·05 per cent.

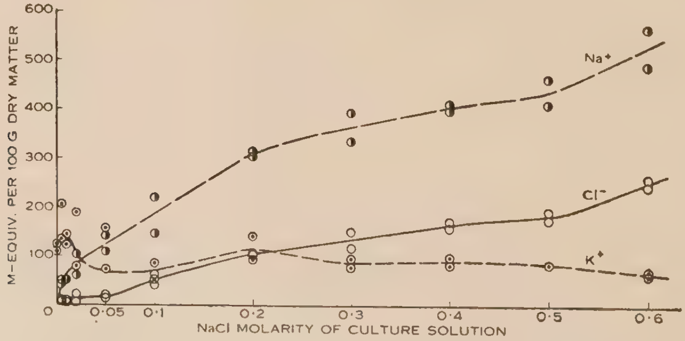

Fig. 4.—Na⁺, K⁺, and Cl⁻ contents of mature leaves of *A. hastata,* plotted on an equivalent/dry matter basis. Sodium and chloride contents for 0 NaCl treatment: Sodium—A = 3, B = 2; chloride—A < 0·3, B = 0·8.

The sulphate level was checked in further samples of the ground mature leaf material by preparing clear aqueous extracts in the same manner as for nitrates, except that cupric chloride was substituted for cupric sulphate. The sulphate present was barely detectable, and its concentration in the cell sap was certainly less than that of the culture solution (4 m-equiv./l).

All analyses were expressed on an oven dry weight basis (dry matter), or on the basis of the difference between the fresh weight and the oven dry weight of the mature leaves, or by both methods. The latter type of expressions were assumed to be a measure of the mean cell sap concentrations of the leaf.

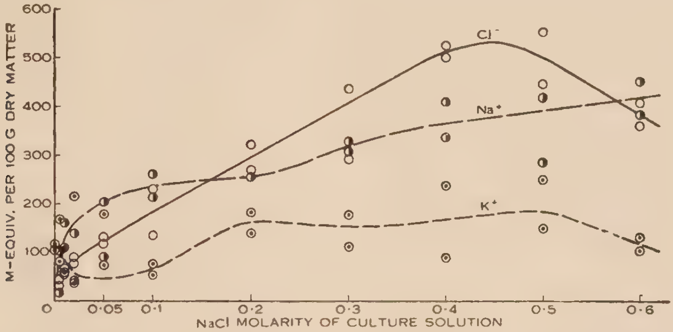

Fig. 5.—Na⁺, K⁺, and Cl⁻ levels of young samples from root systems of *A. hastata*. Sodium and chloride contents for 0 NaCl treatment: Sodium—A = 4, B = 6; chloride—A = 1·7, B = 1·4.

III. Analytical Results

The chloride results showed marked differences in the physiological responses of the three species tested (Figs. 1 and 2). The *A. vesicaria* results indicated accumulation* of NaCl over the whole range. The chloride percentages at the 0·3 and 0·4M NaCl levels are appreciably higher than any known value for field specimens. The growth at these high levels was little affected (Plate 1, Fig. 1).

The tomato plants, which as seedlings possessed the least tolerance to NaCl, occupied an intermediate position as far as chloride uptake was concerned (Figs. 1 and 2).

The *A. hastata* seedlings were tolerant to NaCl concentrations approximating that of sea-water, but their subsequent growth at these high concentrations was markedly inhibited (Fig. 8; Plate 1, Fig. 3). The chloride uptake of their mature leaves was very low compared both with the other species used and the external chloride concentrations (Figs. 1 and 2). For the root systems, on the other hand, the chloride uptake on a dry weight basis was much greater, but showed a falling off at the highest level where the saline toxicity symptoms were greatest (Fig. 3).

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* In this paper Robertson's (1951) definition of accumulation is adopted. In this sense the term implies the concentrating of both ions of a salt against a concentration gradient, and also the expenditure of energy by the cell.

When the dominant ionic constituents of the *A. hastata* leaves were plotted on a dry matter basis as equivalents (Fig. 4), it became apparent that over the entire range the total inorganic cations probably greatly exceeded the total inorganic anions. On the other hand, a similar treatment for the root systems (Fig. 5) showed that an approximate balance probably existed at least in the central portion of the range.

In both these latter treatments, high potassium and low sodium values were recorded for three dwarfed plants in the low-salt portion of the range. These three plants, for reasons unknown, failed to enter into the period of rapid growth prior to the onset of flowering, which is characteristic of the species. In both mature leaves and roots this resulted in higher potassium values, while in the roots only, there was a corresponding drop in the sodium values. Potassium dilution in the culture solutions, brought about by the larger plants, probably caused these effects. These figures were omitted from consideration in plotting the results (Figs. 4 and 5). In the leaves these high potassium values ceased to be as significant when plotted on a cell sap basis (Figs. 6 and 7).

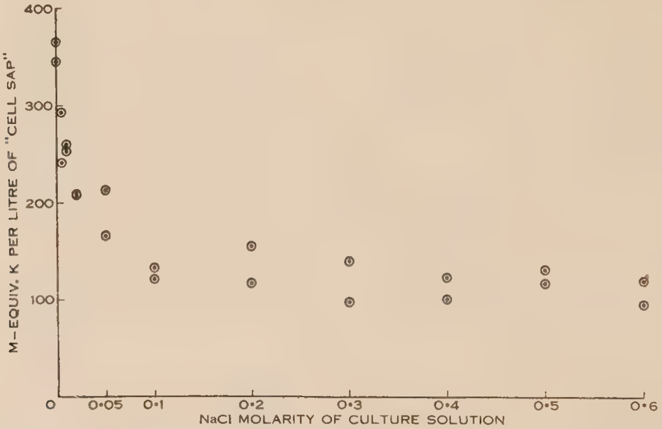

Fig. 6.—Concentrations of K on a leaf moisture basis for the mature leaves of *A. hastata*.

The mutual competition exhibited between the sodium and potassium ion uptakes was most clearly illustrated by expressing the potassium of the mature leaves on an equivalent cell sap basis (Fig. 6). This was because the addition of only small amounts of NaCl to the culture solutions caused a marked increase in the water/dry matter ratio of the leaves. However, the effect is quite noticeable for the potassium values on a dry matter basis (Fig. 4), when the three dwarfed plants in the low-salt treatments are compared with the dwarfed plants in the high-salt treatments.

The plotting of values for the total equivalents of sodium and potassium with those for chloride on a cell sap basis for the mature leaves (Fig. 7), indicated the uniform nature of the anionic deficit when the chloride ion only is considered. The agreement of duplicates was also rather better in this treatment.

In order to obtain more information on the anion composition of the leaves, determinations of water-soluble phosphates, nitrates, and sulphates were carried out. The sulphate ion concentration of the cell sap was certainly less than that of the external media (4 m-equiv./l), and thus plays no important part in the cation-anion balance.

The phosphate ion concentration was appreciable in many instances, and was generally higher in the smaller plants (Fig. 8). This effect was probably caused by a dilution of the phosphate in the culture solution by the larger plants.

Nitrate ion concentrations for the 0, 0·1, 0·2, 0·3, 0·4, 0·5, and 0·6M NaCl treatments are shown with the ionic concentrations already mentioned plus levels of calcium ions in Figure 8. It can be seen from these summations that although phosphate and nitrate ions may make up an appreciable portion of the total anions present, particularly in the physiologically younger plants, there remains a considerable anionic concentration (up to 500 m-equiv./l). of possibly mainly organic acid radicals (Ulrich 1941).

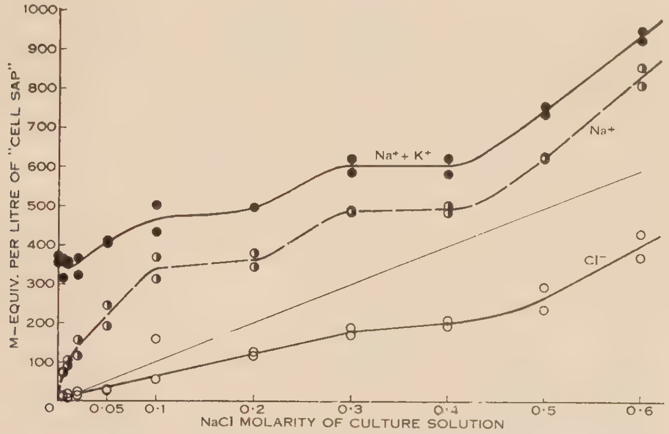

Fig. 7.—Concentrations of Na^+, $Na^+ + K^+$, and Cl^- for the mature leaves of *A. hastata*, expressed on a leaf moisture basis, and compared with the external NaCl concentrations. Sodium and chloride contents for 0 NaCl treatment: Sodium—A = 9, B = 7; chloride—A < 1, B = 2.

In a further consideration of the relative concentrations in the cell sap of the leaf of the sodium and chloride ions, it was thought that these might agree with what would be expected assuming a Donnan equilibrium existed. If this were so, a further assumption would be that these ions were freely diffusible in the leaf, and along a conduction path to the external medium, and that the potentially more important mass movement of ions by the vascular tissue had reached an equilibrium condition, in which the ions entering the leaf via the transpiration stream were balanced by an equal amount leaving the leaf. Such an equilibrium could not be envisaged for young actively growing leaves or roots. The non-diffusible ions of consequence would be the balance of the anions (A^-), and the potassium ions, the latter and some of the former being restrained by accumulation processes. We may represent the equilibrium condition by the following:

Culture Solution (*c*)	Leaf (*l*)
Na^+	K^+
Cl^-	Na^+
	Cl^-
	A^-

At equilibrium we have, from simple kinetic theory, that there must be a balance of the activities of the sodium and chloride ions. This activity is proportional to the concentrations* of both ions. Thus

$$[Na^+]_c \times [Cl^-]_c = [Na^+]_l \times [Cl^-]_l. \quad \ldots\ldots\ldots\ldots(1)$$

To fulfil the condition of electrical neutrality we have

$$[Na^+]_l + [K^+]_l = [Cl^-]_l + [A^-]_l,$$

or

$$[Na^+]_l = [Cl^-]_l + [A^-]_l - [K^+]_l. \quad \ldots\ldots\ldots\ldots(2)$$

From the results (Figs. 7 and 8) we know that

$$[A^-]_l > [K^+]_l.$$

Therefore, from (**2**)

$$[Na^+]_l > [Cl^-]_l, \quad \ldots\ldots\ldots\ldots(3)$$

and from (**1**)

$$[Na^+]_c < [Na^+]_l, \quad \ldots\ldots\ldots\ldots(4)$$

and

$$[Cl^-]_c > [Cl^-]_l. \quad \ldots\ldots\ldots\ldots(5)$$

The inequalities (**3**) and (**4**) agree with all the results obtained (Figs. 7 and 8; Table 1). Inequality (**5**) does not hold for some of the treatments between 0 and 0·1M NaCl inclusive. This indicates that there is some accumulation of NaCl at these levels. Inequality (**5**) does hold quite regularly for all treatments above 0·2M NaCl.

To check the assumption that the sodium and chloride ions were behaving as if freely diffusible, equation (**1**) was tested by comparing the initial and final chloride concentration squared, of the culture solutions, with the product of the sodium and chloride concentrations in the cell sap of the mature leaves (Table 1). These comparisons show reasonable agreement from the 0·2 to 0·6M NaCl levels inclusive. Once again at the NaCl levels under 0·2M there is evidence of accumulation of these ions. Thus for the higher portion of the range, there is substantial agreement between the results obtained, and what would be expected assuming a Donnan equilibrium existed between the mature leaves and the external culture solution.

At this stage it was considered important to know something of the ionic concentrations existing in the young growing leaves, and thus obtain some information on the main trends of these concentrations in the ontogeny of the leaf. Sampling was not complete because in the larger plants terminal buds had commenced flower production, while in others, the dried material had been damaged, or the plants were too small to allow adequate sampling.

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* For the sake of simplicity concentrations have been used; corrections to be made for activity are less than the probable sampling errors in collecting the data.

The results for the young leaves and buds (Table 2), indicate that for the actively growing plants in the culture solutions up to 0·3M NaCl, the chloride contents may be 2–3 times that of the mature leaves of the same plant. However, potassium contents in these regions are up to 30 per cent. less than in the mature leaves, and sodium values also are in general slightly lower. In spite of these differences, the dominance of the total cations over the chloride ions appears to be maintained in the young leaves and buds.

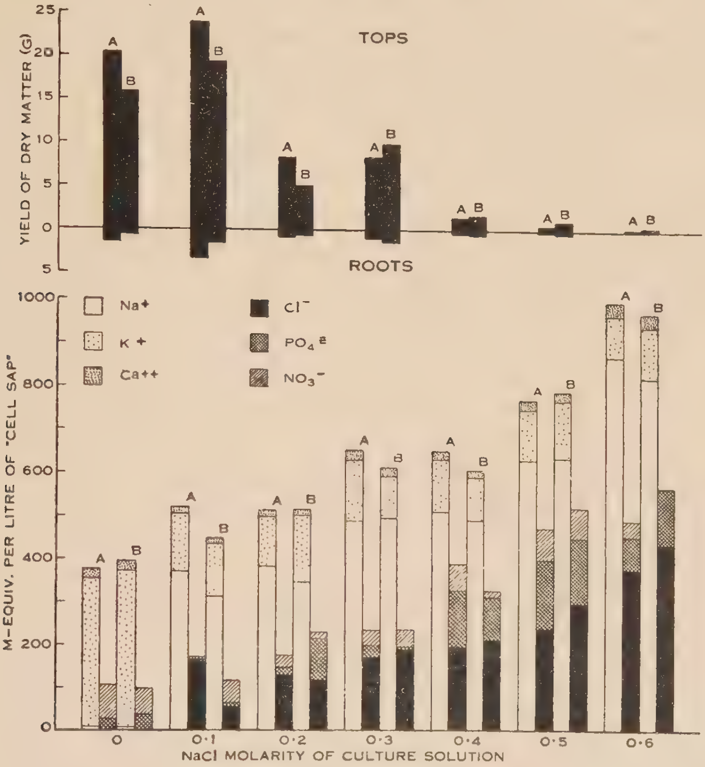

Fig. 8.—Summations of the inorganic cations and anions determined in the aqueous extracts of the mature leaves of *A. hastata*, together with yields of roots and tops for the corresponding plants. Separate values for the duplicate series A and B are presented side by side. Treatments between 0 and 0·1M NaCl are not shown. A nitrate analysis on 0·6M B was not done.

IV. Discussion and Conclusions

It is clear from the chloride-uptake results for mature leaves (Figs. 1 and 2), that the two species of *Atriplex* react to the problem of coping with a saline substrate in two very different ways, the mesophytic tomato plants, which do not possess any tolerance of very saline conditions, following an intermediate course. *A. vesicaria* is better adapted for growth than *A. hastata* under the conditions of highly saline water cultures (Plate 1, Figs. 1 and 3). *A. hastata*, being an annual, is probably well adapted to the fluctuating salinity conditions typical of moist coastal salt-marshes and strands. Thus its relatively vigorous growth could be completed in short periods after rain, when the salinity conditions were most favourable.

TABLE 1

COMPARISON OF INITIAL AND FINAL CHLORIDE CONCENTRATION* OF CULTURE SOLUTIONS WITH SODIUM AND CHLORIDE CONCENTRATIONS IN CELL SAP OF MATURE LEAVES OF A. HASTATA

Series	Culture Solutions				Mature Leaves		
	Initial		Final				
	$[\mathrm{Cl}^-]$	$[\mathrm{Cl}^-]^2$	$[\mathrm{Cl}^-]$	$[\mathrm{Cl}^-]^2$	$[\mathrm{Na}^+]$	$[\mathrm{Cl}^-]$	$[\mathrm{Na}^+]\times[\mathrm{Cl}^-]$
A	0	0	0·012	$14{\cdot}4\times10^{-5}$	9	1	9
B	0	0	0·004	$1{\cdot}6\times10^{-5}$	7	2	14
A	5	25	4·66	21·7	72	15	1080
B	5	25	3·87	15·0	75	12	900
A	10	100	7·60	57·8	105	19	1990
B	10	100	8·54	73·0	90	7	630
A	20	400	15·0	225	157	14	2200
B	20	400	18·7	350	115	24	2800
A	50	$2{\cdot}5\times10^{3}$	43·8	$1{\cdot}92\times10^{3}$	190	28	$5{\cdot}3\times10^{3}$
B	50	$2{\cdot}5\times10^{3}$	45·4	$2{\cdot}06\times10^{3}$	240	30	$7{\cdot}2\times10^{3}$
A	100	1×10^{4}	85·6	$0{\cdot}732\times10^{4}$	370	160	$5{\cdot}9\times10^{4}$
B	100	1×10^{4}	92·8	$0{\cdot}862\times10^{4}$	310	57	$1{\cdot}8\times10^{4}$
A	200	4×10^{4}	190	$3{\cdot}61\times10^{4}$	380	128	$4{\cdot}9\times10^{4}$
B	200	4×10^{4}	195	$3{\cdot}80\times10^{4}$	350	117	$4{\cdot}1\times10^{4}$
A	300	9×10^{4}	289	$8{\cdot}35\times10^{4}$	490	171	$8{\cdot}4\times10^{4}$
B	300	9×10^{4}	295	$8{\cdot}70\times10^{4}$	490	190	$9{\cdot}3\times10^{4}$
A	400	16×10^{4}	378	$14{\cdot}3\times10^{4}$	510	195	$9{\cdot}9\times10^{4}$
B	400	16×10^{4}	388	$15{\cdot}0\times10^{4}$	490	211	$10{\cdot}3\times10^{4}$
A	500	25×10^{4}	477	$22{\cdot}7\times10^{4}$	630	239	$15{\cdot}1\times10^{4}$
B	500	25×10^{4}	494	$24{\cdot}4\times10^{4}$	640	298	$19{\cdot}1\times10^{4}$
A	600	36×10^{4}	556	$30{\cdot}9\times10^{4}$	870	375	33×10^{4}
B	600	36×10^{4}	608	$36{\cdot}9\times10^{4}$	820	436	36×10^{4}

* All concentrations are in m-equiv./l.

The position of *A. vesicaria* as a xerophyte is of interest because of its extremely high uptake of chloride ions (Wood 1925; Black 1951). Recent analyses (Whalley and Gibson, personal communication) on a number of field samples of *A. vesicaria* leaves, have shown that in practically all cases these extremely high chloride contents are adequately balanced by sodium and potassium ions. In 20 samples the sodium uptakes averaged five times the potassium uptakes on an equivalent basis.

The low absorption of chlorides as shown here for *A. hastata* is by no means typical of halophytes. In the same genus, *A. paludosa* R.Br., an indigenous perennial species found commonly in the drier coastal salt-marshes of southern Australia, has been reported by Wood (1925) to have up to 18·9 per cent. chloride of dried leaf material. Keller (1925) has shown that *Salicornia herbacea* L., when grown in sand culture treated with a solution containing 4·1 per cent. NaCl, had values of 50 per cent. ash and 26 per cent. chloride for the dry weight of the tops. Using the same species, van Eijk (1939) recorded 18 per cent. chloride of dry matter for plants grown in a 3 per cent. NaCl solution. All the above examples are taken from halophytes grown in highly saline growth media. It has been claimed that halophytes absorb more chloride, even from non-saline soils, than non-halophytes from the same soil (Steiner 1939).

TABLE 2

SODIUM, POTASSIUM, AND CHLORIDE CONTENTS OF MATURE LEAVES AND YOUNG LEAVES AND BUDS OF A. HASTATA

Results expressed as m-equiv./100 g dry matter

Series	Treatment (NaCl molarity of culture solution)	Mature Leaves			Young Leaves and Buds		
		Sodium	Potassium	Chloride	Sodium	Potassium	Chloride
A	0·005	50	205	10·2	39	140	27·4
A	0·2	300	93	102	270	74	199
A	0·3	330	95	117	300	74	244
A	0·4	410	98	157	420	74	187

Among the alkali and alkaline earth cations, sodium is the cation which responds most to specific variations in plants (van Itallie 1935; Collander 1941; Takada 1954). *A. hortense* L., *A. litorale* L., and *Salicornia herbacea* were shown by Collander (1941) to absorb unusually great totals of cations, including larger amounts of sodium than found in other non-halophytic species growing in the same culture solutions. Notwithstanding these facts, when culture solutions containing cations in equivalent concentrations are considered, sodium generally is the one least absorbed or most perfectly excluded (Collander 1941). The values for sodium and potassium uptakes of *A. hastata* at the 0·005M NaCl level (approximately equivalent to the potassium concentration) agree with the latter finding (Table 3).

The mutual competition between sodium and potassium uptakes evident from the 0 to the 0·1M NaCl culture levels (Figs. 6, 7, and 8) is significant but by no means complete, as the potassium content remains practically constant for higher NaCl culture levels, instead of falling away to extremely small values. This partial competition between sodium and potassium uptakes agrees with the observations of Collander (1941). The potassium levels represented in Figure 6 show the importance of expressing results on a moisture or cell sap basis.

According to Collander (1941), complete mutual competition was shown by absorption from solutions containing K, Rb, and Cs, or Ca and Sr, in equivalent amounts. He suggests that plants are unable to "distinguish" between these chemically related elements insofar as the salt-uptake mechanism is concerned. However, this does not mean that these ions are of equal metabolic value to the plant, although the non-essential elements may partially substitute for the essential element for some purposes.

This latter consideration should also hold for the mutual competition shown to exist here between the sodium and potassium uptakes of *A. hastata.* Thus from the 0·005M to the 0·1M NaCl culture levels, sodium possibly partially substitutes for potassium in the organic acid buffer complex. Above 0·1M NaCl, potassium contents are fairly constant, and sodium comes into increasing importance as a cell sap cation involved in the maintenance of an osmotic pressure equivalent to the osmotic pressure of the external culture solution.

Table 3

COMPARISON OF SODIUM AND POTASSIUM UPTAKES FOR THREE SPECIES OF ATRIPLEX GROWN IN CULTURE SOLUTIONS OF APPROXIMATELY EQUIVALENT SODIUM AND POTASSIUM CONCENTRATIONS

Series	Plant Species	Culture Solution Concentrations (m-equiv./l)		Dried Plant Material (m-equiv./100 g)	
		Sodium	Potassium	Sodium	Potassium
A	*A. hastata*—mature leaves (dwarfed plant)	5	6	50	205
B	*A. hastata*—mature leaves	5	6	41	133
—	*A. hortense**—whole plant	4	4	94	187
—	*A. littorale**—whole plant	4	4	46	243

* Results for these species calculated from Collander (1941).

Mutual competition between sodium and potassium uptakes by the tops of four species grown in a mixture of sand and synthetic ion-exchange resins are shown in results obtained by Bower and Wadleigh (1948). In this experiment potassium levels were held constant at 10 per cent. of the cation exchange capacity, while sodium varied according to treatments, from 0 to 75 per cent. of the cation exchange capacity. The decreasing potassium uptakes corresponding to increasing sodium uptakes were interpreted as being caused by a lowered exchangeability of potassium on the resin colloids, as the adsorbed complementary ions moved from calcium and magnesium to sodium. This explanation does not appear to be necessary, as the same phenomenon occurs with plants growing in water cultures.

Of all the inorganic ions measured quantitatively in the mature leaves of *A. hastata*, only the Cl^- and SO_4^{--} ions were present in concentrations below the external culture solution concentrations. It has been shown that from the 0·2 to 0·6M levels inclusive, the uptake of Na^+ and Cl^- ions is as would be expected, assuming a Donnan equilibrium existed between the mature leaves and the external culture solution. However, this does not necessarily mean that these ions had never been subjected to accumulation processes in the plant. In the root systems of the plants from the Donnan-agreement range, the chloride levels were much higher than in the leaves, and there was an approximate balance between the Cl^- ions and the Na^+ and K^+ ions (Figs. 3 and 5). Thus at this level of the plant the Donnan effects are masked, possibly by NaCl accumulation against a concentration gradient. The drop in the chloride content of the roots in the 0·6M NaCl culture solutions is possibly a response to a lowered metabolic activity. Such a region of salt accumulation in the roots may form a side branch to the main stream of salt uptake in these plants.

The work of Steward (1935, 1948) and Steward and Millar (1954) has stressed the importance in salt uptake, of the young developing leaves in the dicotyledonous shoot system. In these regions, high accumulation rates are associated with the high metabolic activities which occur during periods of active growth by cell enlargement. In the data for the young leaves and buds of *A. hastata* (Table 2), the chloride contents only are appreciably higher than in the mature leaves, and this effect is more marked in those plants in an active state of vegetative growth (Fig. 8; Plate 1, Fig. 3). However, the decrease in immobile anions (A^-), together with the increase in activity of the Na^+ and Cl^- ions on a dry weight basis, points to accumulation of NaCl in these regions of high metabolic activity, although the evidence is not conclusive. It is probable, then, that much of the salt present in the mature leaf is absorbed by an accumulation mechanism during the early stages of its ontogeny.

Another mechanism of salt uptake in the plant that must be considered, is the mass movement of ions in the xylem due to the transpiration stream. In the mature leaf, the salt entering the leaf in this way, must be balanced by an approximately equal amount leaving the leaf, probably via the phloem.

It has long been recognized that a simple Donnan equilibrium is inadequate to explain even the salt uptake of pieces of plant tissue placed in a solution of the salt, because a balance of the ionic activities of the external and internal phases is not normally found (Briggs and Petrie 1928). However, in this experiment such a balance has been shown to exist over a wide range of saline water cultures. The supposition of this agreement is that the ionic quantities expressed on a moisture basis, are a measure of the mean cell sap concentrations in the mature leaf. Also differences of concentration which no doubt occur from cell to cell and within the cell are disregarded.

It is clear from these considerations, that it is impossible to postulate the Donnan equilibrium *per se* as a mechanism to explain the sodium and chloride uptakes observed. Nevertheless, for the conditions described, the results appear to be consistent with a Donnan adjustment, though other mechanisms are probably operative.

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VI. References

Best, R. J. (1929).—A rapid electrometric method for determining the chloride content of soils. *J. Agric. Sci.* **19**: 533-40.

Black, R. F. (1951).—The halophilous and xerophytic properties of the genus *Atriplex*. Honours Thesis, University of Sydney.

Black, R. F. (1954).—The leaf anatomy of Australian members of the genus *Atriplex*. I. *Atriplex vesicaria* Heward and *A. nummularia* Lindl. *Aust. J. Bot.* **2**: 269-86.

Bower, C. A., and Wadleigh, C. H. (1948).—Growth and cationic accumulation by four species of plants as influenced by various levels of exchangeable sodium. *Proc. Soil Sci. Soc. Amer.* **13**: 218-23.

Briggs, G. E., and Petrie, A. H. K. (1928).—On the application of the Donnan equilibrium to the ionic relations of plant tissues. *Biochem. J.* **22**: 1071-82.

Collander, R. (1941).—Selective absorption of cations by higher plants. *Plant Phys.* **16**: 691-720.

van Eijk, M. (1939).—Analyse der Wirkung des NaCl auf die Entwicklung, Sukkulenz und Transpiration bei *Salicornia herbacea*, sowie untersuchungen über der Einfluss der Salzaufnahme auf die Wurzelatmung bei *Aster tripolium*. *Rec. Trav. Bot. Néerl.* **36**: 559-657.

Hoagland, D. R., and Arnon, D. I. (1938).—The water-culture method for growing plants without soil. Circ. Calif. Agric. Expt. Sta. No. 347. pp. 1-39.

van Itallie, Th. B. (1935).—The role of sodium in the cation-balance of different plants. Trans. 3rd Int. Congr. Soil Sci. Vol. I. pp. 191-4.

Keller, B. (1925).—Halophyten-Xerophytenstudien. *J. Ecol.* **13**: 224-61.

Osborn, T. G. B., and Wood, J. G. (1923).—On some halophytic and non-halophytic plant communities in arid South Australia. *Trans. Roy. Soc. S. Aust.* **47**: 388-99.

Robertson, R. N. (1951).—Mechanism of absorption and transport of inorganic nutrients in plants. *Annu. Rev. Pl. Physiol.* **2**: 1-24.

Steiner, M. (1939).—Die Zusammensetzung des Zellsaftes bei höheren Pflanzen in ihrer ökologischen Bedeutung. *Ergebn. Biol.* **17**: 151-254.

Steward, F. C. (1935).—Mineral nutrition of plants. *Annu. Rev. Biochem.* **4**: 519-44.

Steward, F. C. (1948).—Metabolism and salt uptake by plants. Some problems and precepts for the application of radioactive isotopes. Conf. Rep. Brookhaven Lab. Rept. No. BNL-C-4. pp. 94-103.

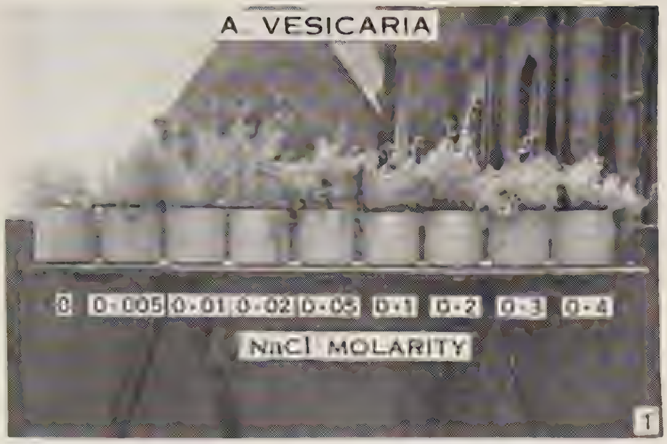
Steward, F. C., and Millar, F. K. (1954).—Salt accumulation in plants: a reconsideration of the role of growth and metabolism. *Symp. Soc. Exp. Biol.* **8**: 367-406.

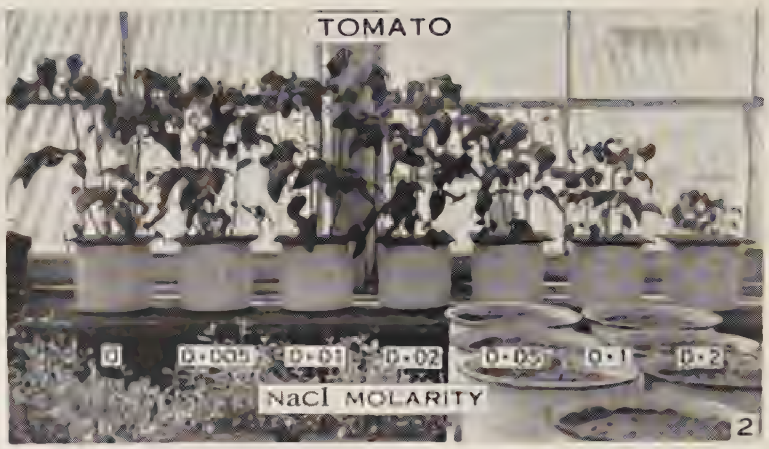
Takada, H. (1954).—Ion accumulation and osmotic value of plants, with special reference to strand plants. *J. Inst. Polyt. Osaka* **5**: 81-96.

Ulrich, A. (1941).—Metabolism of non-volatile organic acids in excised barley roots as related to cation-anion balance during salt accumulation. *Amer. J. Bot.* **28**: 526-37.

Wood, J. G. (1925).—The selective absorption of chlorine ions, and the absorption of water by the leaves in the genus *Atriplex*. *Aust. J. Exp. Biol. Med. Sci.* **2**: 45-56.

ION UPTAKE AND GROWTH OF ATRIPLEX HASTATA





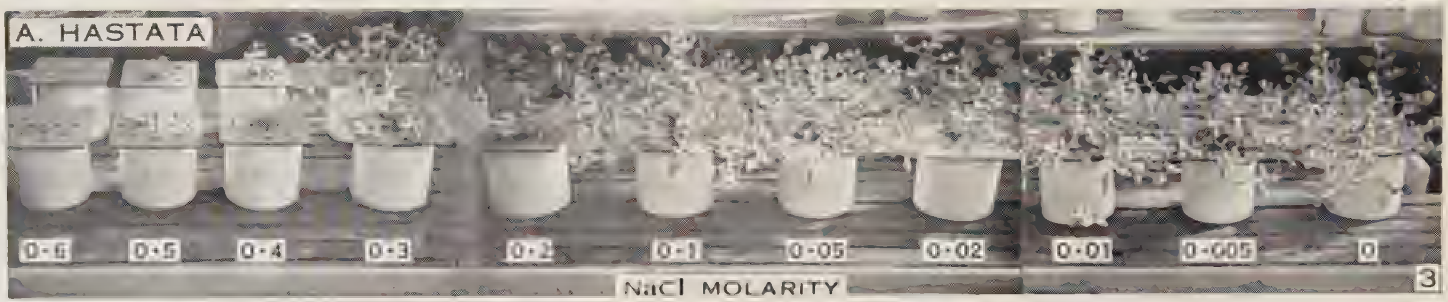


Fig. 1.—*A. vesicaria* series after 25 weeks of growth from seedling stage, and just prior to sampling.

Fig. 2.—Tomato series after 4 weeks of growth from transplantation of seedlings, and at time of sampling.

Fig. 3.—Duplicate *A. hastata* series after 16 weeks of growth from seedling stage, and just prior to harvesting. Series B is in the foreground.

ENZYMIC ACTIVITIES OF SUB-CELLULAR PARTICLES FROM LEAVES

I. THE OCCURRENCE OF MITOCHONDRIA IN GREEN LEAVES OF THE PEA PLANT

By R. M. Smillie*

[*Manuscript received July* 21, 1955]

Summary

Cytoplasmic particles corresponding to the mitochondria of animal and etiolated plant tissues have been prepared from green pea leaves. These particles contain the complete tricarboxylic acid cycle complex of enzymes. This has been determined by demonstrating the oxidative and phosphorylative capacities of the particles in the presence of added substrates of the tricarboxylic acid cycle, the "sparked" oxidation of pyruvate, the conversion of fumarate to succinate in the presence of malonate, and by the complete oxidation of added substrate. Enzymes capable of oxidizing formate, lactate, a number of amino acids, and reduced cytochrome *c* are located in these particles. Diphosphopyridine nucleotide, triphosphopyridine nucleotide, and succinate-linked cytochrome reductases are also present. The effects of various cofactors and inhibitors on the oxidative capacities of the particles have been investigated. Values between 2 and 3 for the ratio of phosphorus esterified to oxygen consumed have been obtained for all the tricarboxylic acid cycle substrates tested, with the exception of succinate, for which a value of 1·58 was found.

I. Introduction

Previous studies have established beyond doubt the existence of mitochondria, defined by certain biochemical activity, in the cells of seedlings of certain etiolated plants (Millerd and Bonner 1953). With green tissue however, the position is quite obscure. Brummond and Burris (1954) have made an extensive study of the location of enzymes of the tricarboxylic acid cycle in both the leaves and cotyledons from light-grown seedlings of the white lupin. Although most of the enzymes of the tricarboxylic acid cycle were demonstrated in extracts of acetone-dried powders of these tissues, the only activity associated with particles of mitochondrial size, isolated by the usual procedures employed in the preparation of plant mitochondria, was an oxidation of α-ketoglutarate. Using identical preparative procedures, active mitochondria were obtained from cotyledons of dark-grown white lupins. Further, it was observed that a progressive decrease in the enzymic activity of the particles from cotyledons occurred, when etiolated seedlings were exposed to light for increasing periods of time. In addition to α-ketoglutarate oxidase, cytochrome oxidase (McClendon 1953; Jagendorf and Wildman 1954), oxalic oxidase (Arnon and Whatley 1954), and catalase (Jagendorf and Wildman 1954) have been associated with non-chloroplastic cytoplasmic particles from green leaves.

In a previous study on the distribution of mitochondria in the pea plant (Smillie 1955), it was shown that particulate preparations from the hull and seed of mature plants, and from the stem and leaf of light-grown seedlings, could oxidize

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certain members of the tricarboxylic acid cycle. In the present paper the properties of cytoplasmic particles obtained from the leaf of green pea plants are investigated and it will be shown that these preparations possess the criteria of biochemically active mitochondria, namely the tricarboxylic acid cycle, the pyridine nucleotide-cytochrome system, and the mechanism for oxidative phosphorylation (Green 1954).

II. METHODS

(*a*) *Plant Material*

Pea plants, *Pisum sativum* (Yates Greenfast), were grown under glasshouse conditions for 10-20 days. Cytoplasmic particles were isolated from the leaves as previously described (Smillie 1955).

(*b*) *Measurement of Enzyme Activity*

Oxygen uptake was measured using the conventional Warburg apparatus, all experiments being conducted at 30°C. Dehydrogenase activity was measured by photometrically following the decrease in optical density of 2, 6-dichlorophenol-indophenol, under anaerobic conditions, in the presence of the appropriate substrate. Cytochrome oxidase was demonstrated by following the decrease in optical density at 550 mμ in the presence of reduced cytochrome *c*. Similarly, cytochrome reductase was assayed by the increase in optical density at 550 mμ in the presence of oxidized cytochrome *c* and substrate. For this assay, the enzyme and cytochrome *c* were first mixed and the cytochrome oxidase present allowed to fully oxidize the cytochrome *c*. Potassium cyanide was added to inhibit the cytochrome oxidase, and the substrate then added.

The ratio of phosphorus esterified to oxygen absorbed (P: O ratio) was measured as previously described (Smillie 1955).

(*c*) *Chemicals and Enzymes*

A coenzyme concentrate (Coe) from yeast was made as previously described (Smillie 1955). 50 per cent. diphosphopyridine nucleotide (DPN), 30 per cent. triphosphopyridine nucleotide (TPN), and glucose-6-phosphate dehydrogenase were prepared as described by Le Page and Mueller (1949). Alcohol dehydrogenase was prepared according to Racker (1950) and hexokinase according to Berger *et al.* (1946) (to step IIIA). *Iso*citrate was made from the lactone and *cis*-aconitate from *trans*-aconitic acid (Krebs and Eggleston 1944). The method of Keilin and Hartree (1937) was employed to prepare cytochrome *c*, which was reduced when required by hydrogen in the presence of activated palladium. Inosinic acid was made by Lehninger's (1951) method and *N*-methylphenazine sulphate by methylation of phenazine with dimethyl sulphate (Kehrmann and Havas 1913) and recrystallization from alcohol/ether mixture.

III. RESULTS

(*a*) *Description of Particles*

The particulate preparations from pea leaves contained considerable amounts of green material, the chlorophyll to total nitrogen ratio being in the vicinity of 0·6. Examination with a Zeiss phase-contrast microscope showed that they consisted of birefringent particles, 0·5-2·0 μ in diameter. Whole chloroplasts and the larger chloroplast fragments were almost completely removed by the initial low speed centrifugation, but many of the remaining smaller particles, as far as could be seen, appeared to be irregular in shape, suggesting that they were small fragments of broken chloroplasts. Rupture of the leaf cells by grinding with sand, resulted in considerable damage to chloroplast structure, and because of this, it was impossible to obtain by differential centrifugation chlorophyll free fractions consisting of particles smaller than whole chloroplasts. In addition, particularly in young leaves, there are probably many small plastids, which would be sedimented with the mitochondrial fraction.

TABLE 1

ANAEROBIC OXIDATION OF VARIOUS SUBSTRATES BY PEA LEAF PARTICLES USING 2,6-DICHLOROPHENOLINDOPHENOL AS THE HYDROGEN ACCEPTOR

The complete system contains enzyme (0·2 ml), tris(hydroxymethyl)-aminomethane buffer pH 7·2 (0·01M), $MgSO_4$ (0·001M), Coe (1 mg), substrate (0·02M), and 2,6-dichlorophenolindophenol ($3{\cdot}3 \times 10^{-5}$M). Final volume 6·0 ml. Results are expressed as per cent. rate of oxidation of malate, which is arbitrarily taken as 100

Experiment	Substrate	Rate	Substrate	Rate
1	Malate	100	*a*-Ketoglutarate	74
	None	24	*iso*Citrate	104
	Fumarate	78	*cis*-Aconitate	96
	Succinate	97	Citrate	115
2	Malate	100	Formate	102
	None	11	Lactate	47
	Oxalate	11		
3	Malate	100	Aspartate	48
	None	13	Asparagine	31
	Serine	27	Glutamate	47
	Glycine	42	Glutamine	48
	Alanine	45	Proline	34

(*b*) *Dehydrogenases Present in the Particulate Preparation*

By using Thunberg tubes and 2, 6-dichlorophenolindophenol as the hydrogen acceptor, it was possible to demonstrate the anaerobic oxidation of a number of substrates by enzymes present in the particulate preparation. Citrate, *cis*-aconitate, *iso*citrate, *a*-ketoglutarate, succinate, fumarate, and malate were all oxidized. In addition, formate, lactate, and a number of amino acids were also oxidized (Table 1). The results in Table 1 are expressed as the per cent. rate of oxidation compared with the oxidative rate with malate as substrate, which is arbitarily taken as 100. With malate, a decrease in optical density at 600 mμ of 0·15-0·20/mg N/min occurred during the first 5 min.

Under aerobic conditions it was possible to replace 2, 6-dichlorophenolindophenol by a number of easily reversibly oxidized dyes. Table 2 shows the abilities of *N*-methylphenazine sulphate, tolylene blue, methylene blue, and *o*-bromophenolindophenol to act as hydrogen acceptors during the aerobic oxidation of succinate and *iso*citrate. The reactions, although quite linear for at least 90 min, proceeded quite slowly, the rate of succinate oxidation being less than 1/20 the activity of the succinoxidase system alone. These experiments show that the necessary dehydrogenases for the operation of the tricarboxylic acid cycle, as well as aconitase and fumarase, are present in the preparation.

Table 2

AEROBIC OXIDATION OF SUCCINATE AND ISOCITRATE BY PEA LEAF PARTICLES IN THE PRESENCE OF CERTAIN HYDROGEN ACCEPTORS

The complete system contains enzyme (0·4 ml), tris(hydroxymethyl)-aminomethane buffer pH 7·2 (0·01M), $MgSO_4$ (0·001M), Coe (1 mg), substrate (0·02M), and hydrogen acceptor (1 mg). Final volume 2·0 ml

Hydrogen Acceptor	Oxygen Uptake* (μl O_2/mg N/hr)	
	Succinate	*iso*Citrate
N-Methylphenazine sulphate	23	52
Tolylene blue	22	24
Methylene blue	27	41
o-Bromophenolindophenol	26	43

* Corrected for endogenous oxygen uptake.

(*c*) *Oxidation of Intermediates of the Tricarboxylic Acid Cycle*

The pea leaf particulate preparations exhibited an ability to oxidize all members of the tricarboxylic acid cycle tested. Malate, fumarate, succinate, α-ketoglutarate, glutamate, *iso*citrate, *cis*-aconitate, citrate, and pyruvate, the last only in the presence of small amounts of another tricarboxylic acid cycle substrate such as malate, were all oxidized quite rapidly (Table 3). Most of the activities were fairly linear for 1 hr. An exception was the oxidation of pyruvate which decreased markedly with time. In contrast to this, the rate of oxidation of α-ketoglutarate was linear for up to 3 hr. Allowing for the chloroplastic material in these preparations, the oxidative activities compare favourably with those previously obtained for etiolated tissues (Smillie 1955). Expressed on a tissue fresh weight basis, these activities are higher than those obtained from the stems or roots of etiolated or light-grown seedlings of the same age, but are not as high as activities obtained with corresponding preparations from etiolated leaves.

A systematic study of growing conditions and leaf age in relation to oxidative activities of the isolated particles was not undertaken; it was noted, however, that, in contrast to the constancy of preparations obtained from etiolated tissues, considerable variations in some activities did occur. The ability to oxidize *iso*citrate, α-ketoglutarate, glutamate, or succinate was fairly constant for each preparation, but activities obtained with the other tricarboxylic acid substrates, compared with those obtained for *iso*citrate or α-ketoglutarate, appeared to differ with preparations from plants at different stages of growth. If the plants were grown under conditions of fairly high temperature (27-37°C) and very high humidity, they yielded preparations with a high rate of oxygen uptake in the absence of added substrate (oxygen uptake of 300-500 μl O_2/mg N/hr) and poor rates of oxidation of intermediates of the tricarboxylic acid cycle.

TABLE 3

RATE OF OXIDATION OF SUBSTRATES OF THE TRICARBOXYLIC ACID CYCLE BY PEA LEAF PARTICLES

The complete system contains enzyme (0·6 ml), sucrose (0·35M), phosphate pH 7·3 (0·03M), $MgSO_4$ (0·001M), adenylate (0·0005M), glucose (0·02M), hexokinase (0·2 mg), and Coe (1 mg). Final volume 2·0 ml. Experiment 3—hexokinase and glucose omitted

Experiment	Substrate	Concentration	Oxygen Uptake (μl O_2/mg N/30 min)
1	None	—	78
	Fumarate	0·02M	228
	Succinate	0·02M	508
	α-Ketoglutarate	0·02M	351
	Glutamate	0·02M	326
	*iso*Citrate	0·02M	304
2	None	—	62
	DL-Malate	0·04M	367
	*iso*Citrate	0·02M	318
	cis-Aconitate	0·006M	134
	Citrate	0·02M	286
	Pyruvate	0·02M	64
3	None	—	8
	DL-Malate	0·0015M	25
	DL-Malate + Pyruvate	0·0015M 0·02M	256

A further source of variation in the values for the oxygen consumption can be attributed to the quantity of material of chloroplast origin, as this varies with such factors as the age of the leaf and the amount of grinding during the extraction procedure.

(*d*) *Cofactors and Activators*

The effect of a number of possible cofactors and activators on the oxidation of α-ketoglutarate by the pea leaf particles is shown in Table 4. The oxidation is linked to a phosphorylating mechanism (Section III (*h*)) and hence added phosphate and a phosphate acceptor (adenylate) are required. Adenylate can be replaced by inosinate. These substances are required only in catalytic amounts, thus indicating the presence of enzymes breaking down adenosine and inosine triphosphates. In rapidly oxidizing preparations these enzymes become the limiting factor, since a further stimulation of the oxidation can be obtained by the further addition of yeast hexokinase and glucose (see Table 8).

TABLE 4

THE EFFECT OF CERTAIN COFACTORS ON THE OXIDATION OF α-KETOGLUTARATE BY PEA LEAF PARTICLES

The complete system contains enzyme (0·6 ml), sucrose (0·35M), phosphate pH 7·3 (0·01M), $MgSO_4$ (0·001M), adenylate (0·0005M), and α-ketoglutarate (0·02M). Final volume 2·0 ml

Experiment	Additions	Concentration	Oxygen Uptake (μl O_2/mg N/30 min)
1	Complete	—	150
	−α-Ketoglutarate	—	5
	$-MgSO_4$	—	19
	−Phosphate	—	11
	−Adenylate	—	28
	−Adenylate+inosinate	0·0005M	86
	+Coe	0·5 mg/ml	202
2	Complete	—	104
	−α-Ketoglutarate	—	12
	+DPN	0·0005M	110
	+TPN	0·0005M	107
	+DPN+TPN	—	112
	+Diphosphothiamine	200 μg/ml	123
	+DPN+TPN +Diphosphothiamine	—	134
	+Coe	0·5 mg/ml	149
	+Cytochrome *c*	5×10^{-6}M	102

The small requirements for DPN, TPN, and diphosphothiamine do not fully account for the stimulation given by Coe. Magnesium ions were essential, but exogenous cytochrome *c* was not required. Inert protein (recrystallized egg albumin), lysine, or ethylenediaminetetra-acetate, when included in the reaction mixture, failed to increase the oxidative activity. These substances can increase the activity of certain preparations containing tricarboxylic acid cycle enzymes (Altmann and Crook 1953; Bonner 1954; Price and Thimann 1954). Cysteine and reduced glutathione are themselves oxidized by the leaf preparation, and their presence resulted in a decreased rate of oxidation of α-ketoglutarate.

(*e*) *Inhibitors*

Malonate reversibly inhibits the oxidation of succinate by the pea leaf particles (Table 5). Potassium cyanide (0·01M), sodium azide (0·01M), zinc, or cupric ions (both 0·001M) can completely inhibit the oxidation of succinate or α-ketoglutarate.

(*f*) *The Operation of the Tricarboxylic Acid Cycle*

The data presented above show that the enzymes necessary for the operation of the tricarboxylic acid cycle are present in the particulate preparations obtained from green leaves. Evidence obtained showing the operation of the complete cyclic mechanism is presented below.

(i) *The Sparked Oxidation of Pyruvate.*—The pea leaf particles will oxidize pyruvate only in the presence of suboptimal amounts of a member of the tricarboxylic acid cycle (Table 3). If the cycle is operative, pyruvate should condense with oxalacetate to form citrate. This formation of citrate during the oxidation of pyruvate was demonstrated as follows. Pea leaf particles (0·6 mg N) were incubated for 20 min at 30°C in 2·0 ml of solution containing pyruvate (0·02M), DL-malate (0·0015M), sucrose (0·35M), phosphate at pH 7·3 (0·01M), $MgSO_4$ (0·001M), adenylate (0·0005M), and Coe (1 mg). Controls, in which pyruvate or malate were omitted, were also incubated. After deproteinization, the reaction mixture was chromatogrammed two dimensionally using butanol-formic acid (Lugg and Overell 1948) as the first solvent and ethanol-ammonia (Long, Quayle, and Stedman 1951) as the second solvent. Whatman No. 1 papers were used and the positions of the acids were located by spraying with universal indicator at pH 9-10 in 95 per cent. alcohol. Citrate (and also possibly *iso*citrate, since this compound possesses almost the same R_F under the conditions used) and a smaller amount of aconitate were identified. These results show that pyruvate can condense with the product of malate oxidation to give citrate, which is then further oxidized to the other members of the tricarboxylic acid cycle.

TABLE 5

INHIBITION OF SUCCINATE OXIDATION BY MALONATE

The reaction mixture contains enzyme (0·6 ml), sucrose (0·35M), phosphate (0·01M), $MgSO_4$ (0·001M), adenylate (0·0005M), and succinate (0·01M). 0·2 ml of succinate (2M) added from side-arm 15 min after initial reading

Additions	Oxygen Uptake (μl O_2/mg N)	
	First 15 Min	Second 15 Min
—	144	93
Malonate (0·001M)	62	78
Malonate (0·004M)	25	69

(ii) *The Formation of Succinate from Fumarate in the Presence of Malonate.*—In the complete cycle the series of steps involving the conversion of fumarate to succinate represents almost one complete turn of the cycle. The presence of the additional step to complete the cycle, i.e. the oxidation of succinate to fumarate, is indicated by the data already presented. This step may be inhibited by malonate (Table 5).

Hence the presence of fumarate and malonate should result in an accumulation of succinate if the cycle is present in its entirety. This was demonstrated chromatographically. Fumarate (0·02M) and malonate (0·005M) were incubated with pea leaf particles (0·6 mg N) in 2·0ml of reaction mixture containing sucrose (0·35M), phosphate at pH 7·3 (0·01M), $MgSO_4$ (0·001M), adenylate (0·0005M), and Coe (1 mg). A control (no fumarate) was also included. After 90 min at 30°C, the reaction mixture was deproteinized and chromatogrammed as described above. Four acids with R_F values corresponding with malonate, malate, fumarate, and succinate were obtained. In the control, only a single compound with an R_F corresponding with malonate was detected. This result was verified using aqueous phenol-formic acid (Wilson, King, and Burris 1954) as the second solvent.

TABLE 6

COMPLETE OXIDATION OF ADDED SUBSTRATE BY PEA LEAF PARTICLES

The reaction mixture contains enzyme (1·5–2·0 mg N), sucrose (0·35M), phosphate (0·01M) pH 7·3, $MgSO_4$ (0·001M), adenylate (0·0005M), and Coe (1 mg). Incubation time approx. 3 hr. Experiment 1 also contains pyruvate (0·02M) and malonate (0·005M)

Experiment	Substrate	Amount of Substrate Added (μmoles)	Oxygen Uptake (μl)		Carbon Dioxide Production (μl)	
			Actual	Theoretical	Actual	Theoretical
1	Fumarate	2·25	101	100		
2	Succinate	2·0	144	156		
3	α-Ketoglutarate	2·0	176	180		
4	α-Ketoglutarate	2·25	204	203	274	280

(iii) *Complete Oxidation of Added Substrate.*—The conversion of fumarate to succinate was quantitative on the basis of the oxygen uptake (Table 6). In this experiment fumarate (0·001M) was incubated with malonate (0·005M) and excess pyruvate (0·02M). The concentration of malonate used was sufficient to completely inhibit the succinoxidase system at the concentration of substrate used. In the absence of added malonate, α-ketoglutarate was completely oxidized to carbon dioxide and water, as determined by the oxygen uptake and carbon dioxide production.

(g) *Cytochrome Oxidase and Reductase*

The presence of cytochrome oxidase and cytochrome reductases linked to DPN, TPN, and succinate were demonstrated by spectrophotometric assays (Table 7). The cytochrome oxidase was not linear and only the initial rate is given. The reductase activities were linear after the first minute until the cytochrome *c* had been approximately 90 per cent. reduced, and the values shown in Table 7 are taken from the linear portion of the rate curve. These assays were done on preparations which had been kept for 24 hr at 0-5°C.

(*h*) *Oxidative Phosphorylation*

The aerobic oxidation of tricarboxylic acid intermediates by the pea leaf particles are coupled with a phosphorylative mechanism resulting in the esterification of inorganic phosphate. In Table 8, values for the P: O ratio with certain substrates are shown. It can be seen that all substrates except succinate gave values between 2 and 3, thus showing that mitochondria from green leaves can phosphorylate just as efficiently as those from etiolated plant (Smillie 1955) or animal (Copenhaver and Lardy 1952) tissues. Some phosphorylation takes place in the absence of added hexokinase, thus indicating the presence of this enzyme on the particles.

Table 7

THE OCCURRENCE OF CYTOCHROME OXIDASE AND REDUCTASE IN THE PEA LEAF PARTICULATE PREPARATION

Reaction mixture contains reduced or oxidized cytochrome *c* ($5{\cdot}2 \times 10^{-6}$ moles/ml) and phosphate pH 7·3 (0·01M). Final volume 3·0 ml

Enzyme System Tested	Additions	Pea Leaf Enzyme (μg N)	Increase in Optical Density per Min at 550 mμ
Cytochrome oxidase	—	22	−0·190*
DPN-linked cytochrome reductase	KCN (0·0002M), Coe (1 mg), and alcohol dehydrogenase	22	0·003
	Above + alcohol (0·1M)	22	0·068
TPN-linked cytochrome reductase	KCN (0·0002M), TPN (0·001M), and glucose-6-phosphate dehydrogenase	22	0·001
	Above + glucose-6-phosphate (0·005M)	22	0·026
Succinate-linked cytochrome reductase	KCN (0·0002M)	220	0·018
	Above + succinate (0·02M)	220	0·118

* Initial rate only.

IV. Discussion

The isolation and characterization from the green leaf of cytoplasmic particles, mitochondrial in nature, dispels previous doubts concerning the nature of respiratory processes in chlorophyll containing tissue. It is clear that the mechanisms concerned with the conversion of a major portion of the energy contained in respiratory substrates, to a form utilizable for cell synthesis, are fundamentally similar to those operating in animal and etiolated plant cells. The leaf particles in general appear to require the same cofactors and are similarly affected by the same inhibitors as are mammalian mitochondria. They contain the complete tricarboxylic acid cycle and associated cytochrome and phosphorylative systems, and can in fact completely oxidize added substrates such as α-ketoglutarate, utilizing most of the energy released during these oxidations for the synthesis of adenosine triphosphate. This ability to oxidize completely added substrate has not previously been shown for plant mitochondria.

TABLE 8

OXIDATIVE PHOSPHORYLATION BY PEA LEAF PARTICLES

Reaction mixture as in Table 3. Substrate concentration 0·02M, except *cis*-aconitate (0·006M)

Experiment	Substrate	Phosphorus Uptake* (atoms × 10^{-6}/25 min)	Oxygen Uptake* (atoms × 10^{-6}/25 min)	P : O
1	Fumarate	12·2	5·2	2·34
	Succinate	40·4	25·5	1·58
	α-Ketoglutarate	41·8	14·3	2·92
	Glutamate	25·8	12·3	2·10
	*iso*Citrate	29·4	10·8	2·76
2	*iso*Citrate	42·8	16·0	2·66
	*iso*Citrate†	13·2	12·9	1·02
	cis-Aconitate	10·6	4·5	2·36
	Citrate	23·6	9·4	2·51
	Ascorbate‡	0	6·3	—

* Corrected for uptake in the absence of added substrate.

† Hexokinase omitted.

‡ Also contains cytochrome *c* (5 × 10^{-6}M).

Of particular interest are the values obtained for the P: O ratios, as these are very similar to values obtained in studies with mammalian mitochondria (Krebs 1954). Similar values have also been obtained for non-green plant tissue, using succinate and α-ketoglutarate as the substrates (Laties 1953; Smillie 1955). The rates of phosphorylation for the pea leaf particles are quite high. In a typical experiment, the rate of phosphate uptake in the presence of succinate was 97 μmoles P/mg N/hr, and in the presence of α-ketoglutarate it was 102 μmoles P/mg N/hr. The high phosphorylative capacity suggests that this process plays a major part in respiration leading to synthetic processes within the cell, and also possibly in photosynthesis. In fact the identification of mitochondrial-like particles in leaf cells raises questions of possible relationships between enzymes of the mitochondrial complex and enzymes involved in photosynthesis. In a subsequent paper it will be shown that the particles containing the tricarboxylic acid cycle complex of enzymes are quite distinct from chloroplasts.

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VI. REFERENCES

ALTMANN, S. M., and CROOK, E. M. (1953).—*Nature* **171**: 76.

ARNON, D. I., and WHATLEY, F. R. (1954).—*Physiol. Plant.* **7**: 602.

BERGER, L., SLEIN, M. W., COLOWICK, S. P., and CORI, C. F. (1946).—*J. Gen. Physiol.* **29**: 279.

BONNER, W. D. (1954).—*Biochem. J.* **56**: 274.

BRUMMOND, D. O., and BURRIS, R. H. (1954).—*J. Biol. Chem.* **209**: 755.

COPENHAVER, J. H., and LARDY, H. A. (1952).—*J. Biol. Chem.* **195**: 225.

GREEN, D. E. (1954).—In "Chemical Pathways of Metabolism." Vol. 1, p. 27. (Ed. D. M. Greenberg.) (Academic Press Inc.: New York.)

JAGENDORF, A., and WILDMAN, S. G. (1954).—*Plant Physiol.* **29**: 270.

KEHRMANN, F., and HAVAS, E. (1913).—*Ber. dtsch. chem. Ges.* **46**: 341.

KEILIN, D., and HARTREE, E. F. (1937).—*Proc. Roy. Soc.* B **122**: 298.

KREBS, H. A. (1954).—In "Chemical Pathways of Metabolism." Vol. 1, p. 109. (Ed. D. M. Greenberg.) (Academic Press Inc.: New York.)

KREBS, H. A., and EGGLESTON, L. V. (1944).—*Biochem. J.* **38**: 426.

LATIES, G. (1953).—*Plant Physiol.* **28**: 557.

LEHNINGER, A. L. (1951).—In "Manometric Methods and Tissue Metabolism." p. 207. (Ed. W. W. Umbreit, R. H. Burris, and J. F. Stauffer.) (Burgess Publ. Co.: Minneapolis.)

LE PAGE, G. A., and MUELLER, G. C. (1949).—*J. Biol. Chem.* **180**: 975.

LONG, A. G., QUAYLE, J. R., and STEDMAN, R. J. (1951).—*J. Chem. Soc.* **1951**: 2197.

LUGG, J. W. H., and OVERELL, B. T. (1948).—*Aust. J. Sci. Res.* A **1**: 98.

MCCLENDON, J. H. (1953).—*Amer. J. Bot.* **40**: 260.

MILLERD, A., and BONNER, J. (1953).—*J. Histochem. Cytochem.* **1**: 254.

PRICE, C. A., and THIMANN, K. V. (1954).—*Plant Physiol.* **29**: 113.

RACKER, E. (1950).—*J. Biol. Chem.* **184**: 313.

SMILLIE, R. M. (1955).—*Aust. J. Biol. Sci.* **8**: 186.

WILSON, D. G., KING, K. W., and BURRIS, R. H. (1954).—*J. Biol. Chem.* **208**: 863.

THE OXIDATION OF HYDROCARBONS BY SOIL BACTERIA

I. MORPHOLOGICAL AND BIOCHEMICAL PROPERTIES OF A SOIL DIPHTHEROID UTILIZING HYDROCARBONS

By J. N. Ladd*

[*Manuscript received May 20, 1955*]

Summary

(1) A description is given of the morphology and biochemical properties of a soil bacterium, *Corynebacterium* sp., able to oxidize aliphatic hydrocarbons.

(2) The rates of oxidation of *n*-hexadecane, *n*-tetradecane, *n*-decane, *n*-octane, 1-hexadecene, 1-tetradecene, and 1-dodecene are measured. R.Q. values for the oxidation of *n*-hexadecane, *n*-tetradecane, and *n*-decane indicate their complete oxidation.

(3) Increased dispersion of *n*-decane by surface-active agents did not increase its oxidation rate. However, an emulsion of *n*-decane, homogenized in a blendor and containing only one-fifth of the amount of hydrocarbon, is oxidized at the same rate as the non-homogenized control.

(4) The organism oxidizes *n*-fatty acids (C_1-C_{10}), *n*-fatty alcohols (C_2-C_{11}), several aliphatic aldehydes, and the higher methyl ketones. It is unable to oxidize the lower ketones or cyclic compounds.

(5) Co-oxidation rates of *n*-decane with its oxidative derivatives show no summation of the respective individual rates.

(6) *n*-Decane oxidation is unaffected by malonate in concentrations causing 50 per cent. inhibition of succinate oxidation. *n*-Decane oxidation is inhibited 76 per cent. by 0·004M fluoroacetate, 100 per cent. by 0·008M cyanide, 90 per cent. by 0·12M azide, 100 per cent. by 0·0001M mercuric ions, and 88 per cent. by 0·0008M iodoacetate. Cysteine (0·008M) completely reactivates cells inhibited by iodoacetate.

(7) Two non-volatile acids, chromatographically identical with lactic and glutaric acids, are produced during the oxidation of *n*-decane. No volatile acids were detected.

I. Introduction

Despite the repeated demonstration of oxidation of aliphatic hydrocarbons by a wide variety of microorganisms (Zobell 1950), little is known about the mechanism(s) involved.

Johnson, Goodale, and Turkevitch (1942) investigated the oxidation of a number of pure aliphatic hydrocarbons as well as of mixtures, by *Bacterium aliphaticum* and species of *Pseudomonas*. No intermediate oxidation products were discovered although the pH dropped from 7·0 to 6·5 in 0·001M phosphate buffer, indicative of acid production. Bushnell and Haas (1941) grew species of *Pseudomonas* and *Corynebacterium* in media containing different commercial hydrocarbons as sole sources of energy. Small decreases in pH of the culture medium and increased ease

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of emulsification of oil in water were assumed to be due to organic acid production during bacterial growth.

Using four strains of pathogenic mycobacteria, Saz (1949) studied the comparative rates of oxidation of the aliphatic hydrocarbons ranging from *n*-hexane to *n*-octadecane as well as their unsaturated and monohydric derivatives. Greater uptakes of oxygen were recorded for the higher homologues, but attempts to find traces of fatty acids or other intermediates failed. Webley and de Kock (1952) tested the ability of *Proactinomyces opacus* to oxidize normal fatty acids and alcohols as well as paraffins. The acids were oxidized whereas some of the lower soluble alcohols were not, due probably to their presence in toxic concentrations.

Failure to detect oxidation products other than carbon dioxide makes it impossible to decide even the carbon atom at which primary attack occurs let alone the mechanism involved. The most promising information so far comes from the work of Berhard, Gloor, and Scheitlin (1952) who fed deuterium-labelled *n*-octadecane to rats. Recovery of stearic and palmitic acids containing deuterium from the body fats and liver lipids suggests terminal oxidation.

The work presented here is a description and identification as far as possible of a soil bacterium, capable of oxidizing aliphatic hydrocarbons and their oxidative derivatives. The effect of inhibitors on hydrocarbon oxidation, and the search by chromatographic methods for end products is also described.

II. MATERIALS AND METHODS

(*a*) *Isolation of Microorganism*

Isolates from Adelaide red-brown earth, previously enriched with kerosene and sump oil, were made by plating soil dilutions on a synthetic medium containing kerosene as the sole source of available carbon. The medium had the following composition: 10 ml kerosene, 3·0 g NH_4NO_3, 1·5 g KH_2PO_4, 0·25 g $MgSO_4{\cdot}7H_2O$, 0·1 g $CaCl_2{\cdot}2H_2O$, trace amounts of Fe^{++}, Zn^{++}, Cu^{++}, Mn^{++}, and MoO_4^{--} ions, 15 g agar, made up to 1 l. with distilled water pH 7·2. The inoculated plates were incubated at 25°C for 3 days. Individual colonies were passaged through the kerosene-agar until a pure culture was obtained. Stock cultures were kept on kerosene-agar at 4°C. Of a number of isolates, the microorganism selected for study gave the best compromise between rate of growth and ability to oxidize hydrocarbons.

(*b*) *Morphology*

Coverslip impressions were taken at intervals from nutrient-agar cultures incubated in a moist chamber at 28°C by the technique of Klieneberger-Nobel (1950). Photomicrographs were recorded with an Officine Galileo Universal camera microscope using the phase-contrast lens under oil immersion and an O.G. green filter (500 mμ). Either Kodak Panatomic X or Ilford HP3 film was employed. Screen magnification was ×1900. The bacterial films were fixed in Bouin's solution and stained with simple Giemsa, or by the technique of Dyar (1947), or of Webb (1954) to detect cell walls. With Dyar's technique better photographs were obtained by omitting to counterstain with methylene blue.

Biochemical tests were made according to Jensen (1934) except the sugar-fermentation experiments which were performed by the more sensitive methods of Clarke and Cowan (1952).

Suspension for respiration studies were grown in flasks of nutrient broth, pH 7·0, for 2-3 days at 26°C on a shaking machine. The cells were harvested by centrifugation, washed twice, and suspended in distilled water. The final suspension usually contained approximately 12·0 mg cell dry wt./ml (hot-air oven, 80°C for 8 hr, cooled in vacuum desiccator), or 1·1 mg total cell N/ml (semi-micro-Kjeldahl digestion). The cell suspensions maintain their original activity after storage for 2 weeks at 4°C.

Respiratory exchanges were measured by the Warburg technique at 25°C, the final volume of each cup contents being 2·5 ml. Because of the immiscibility of the hydrocarbons, they were placed in the main compartment, and the suspension (0·5 ml) added from the side-arm. For the measurement of R.Q. values, three manometers were used for each determination (Umbreit, Burris, and Stauffer 1949). Q_{O_2} values are expressed as μl O_2 consumed/mg cell N/hr.

The extraction and chromatographic procedures for the identification of non-volatile acids were the same as described earlier (Ladd and Nossal 1954). Two solvents were employed for development of paper chromatograms: Alkaline solvent—8 vol. 95 per cent. ethanol, 1 vol. water, 1 vol. 7·5N NH_4OH. Acidic solvent—water-poor phase from 3 vol. *n*-butanol, 6 vol. *iso*butanol, 3 vol. 90 per cent. formic acid, 9 vol. water.

All substrates used in this investigation were purchased from L. Light & Co., except the aliphatic monocarboxylic acids which were obtained from the British Drug Houses Ltd.

III. Results

The bacterium is a member of the saprophytic soil diphtheroids described by Jensen (1934), i.e. *Corynebacterium* sp. The organism undergoes distinct morphological changes during its life-cycle (see Plates 1 and 2). After 8-14 hr incubation in fresh medium, the old cells enlarge to long filaments which show occasional branching. The filaments appear to be multicellular, revealed particularly by Webb's stain. By 24 hr the organism has reached the logarithmic phase of growth with shorter cells occurring in the typical V formation associated with the *Corynebacterium* genus. The bacterial cells continue to decrease in length so that in the decline phase (126 hr) the organisms resemble cocci. At this time a small amount of secondary growth occurs leading to longer cells swollen at one end. The organism is non-motile, Gram-positive in young and old cells, and non-acid-fast even to 1 per cent. acid.

The *Corynebacterium* grows well on asparagine-, glycerine-, nutrient-, dextrose-, and Sabouraud's agar. After 5-6 days incubation at 28°C, the growth is smooth, glistening, and pink. A nutrient-broth culture is uniformly turbid with a fine pink sediment. It uses peptone, asparagine, nitrate, or ammonium phosphate as sources of nitrogen, is catalase positive, reduces nitrate to nitrite, and liquefies gelatine. It does not produce indole from peptone, is unable to hydrolyse starch, or invert sucrose. Acid is produced from glycerine and to a lesser extent from galactose, xylose, and possibly glucose. No acid is produced from maltose, lactose, sucrose, raffinose, mannitol, or dulcitol.

The oxidation rates of saturated and unsaturated aliphatic hydrocarbons by the suspension are shown in Figure 1. Increase in chain length of the saturated compounds increases their rate of oxidation. This is consistently true even for *n*-hexadecane although here the initial rate fell off rapidly after 20 min incubation. *n*-Hexane and *n*-heptane were tested as substrates but manometric readings were made unreliable by their high vapour pressures. *n*-Octadecane remains in the solid state at 25°C, so its rate of oxidation cannot be strictly compared with those of its lower liquid homologues. Over a 5-hr oxidation period, an average Q_{O_2} (N) value of 50 was obtained for this hydrocarbon.

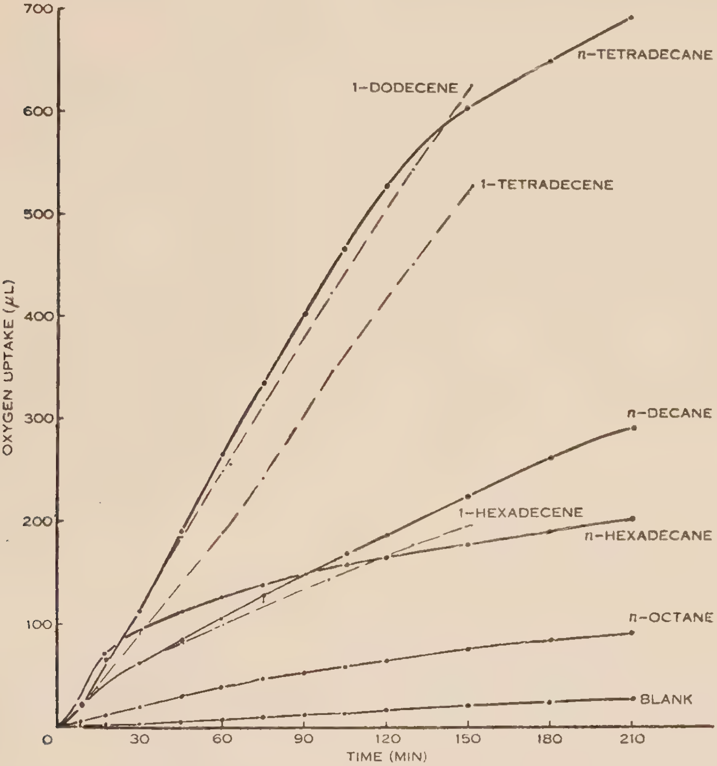


Fig. 1.—Oxidation of saturated and unsaturated aliphatic hydrocarbons. 0·1 ml of each hydrocarbon, 0·03M phosphate buffer pH 7·0, suspension 0·41 mg cell N, cup volume 2·5 ml, bath 25°C.

The hydrocarbons are oxidized without a preliminary lag phase but the oxidation rate is not constant. The initial rate of oxidation exceeds the secondary rate to different degrees depending upon the hydrocarbon. Of the saturated hydrocarbons, this is most marked with *n*-hexadecane and least with *n*-octane. Although not shown in Figure 1, the decline in oxidation rate is true also for the unsaturated derivatives.

Increase in the amount of hydrocarbon increases the oxygen consumption over the range tested (0·1-0·5 ml hydrocarbon per cup). The initial and secondary oxidation rates remain the same but the oxidation period at the higher rate increases with the amount of substrate added.

In contrast with the saturated hydrocarbons, the 1, 2-unsaturated derivatives show a decrease in their rate of oxidation with increasing chain length, except 1-octene which is not oxidized and even inhibits endogenous respiration.

(*a*) *Use of Dispersing Agents*

Because of the insolubility of the hydrocarbons, the use of dispersing agents to promote greater substrate surface for biological attack was investigated. "Tween 80" is itself oxidized by the suspension at a rate greater than that of *n*-decane. Correction for oxidation of the detergent revealed that it neither stimulates nor inhibits *n*-decane oxidation. Sodium desoxycholate is not oxidized, and in a concentration of 0·015 per cent. promotes increased dispersion of *n*-decane. However, hydrocarbon oxidation is not increased.

Emulsions of *n*-decane in buffer were made by homogenizing in a Waring Blendor for 2 min. Although in no case did the emulsions promote greater uptakes of oxygen than an equal amount (0·1 ml) of non-emulsified hydrocarbon, yet equal consumptions were obtained with emulsions containing one-fifth of this amount per cup. This indicates that the enzyme system oxidizing *n*-decane is saturated by 0·1 ml non-emulsified *n*-decane or by 0·02 ml when emulsified. The observed increase in oxygen consumption with increasing amounts of non-emulsified *n*-decane as stated earlier is therefore hard to explain, although it should be pointed out that the actual oxidation rates remain identical.

(*b*) *Oxidation of Cyclic Compounds*

The suspension was tested for its ability to oxidize aromatic hydrocarbons (benzene, toluene, *o*-xylene, *p*-xylene), saturated ring structures (*cyclo*hexane), and partially saturated cyclic hydrocarbons (tetralin), but in no case was a net oxygen uptake observed.

(*c*) *R.Q. Values of the Saturated Hydrocarbons*

The calculated R.Q. values for the oxidation of *n*-decane, *n*-tetradecane, and *n*-hexadecane by the suspension in 0·025M phosphate buffer, pH 7·0, are 0·69, 0·62, and 0·65 respectively. The theoretical R.Q. value for the complete oxidation of a saturated aliphatic hydrocarbon is 0·65.

(*d*) *Oxidation of Saturated Aliphatic Alcohols*

The rates of oxidation of the normal saturated monohydric alcohols from ethanol to *n*-undecyl alcohol are shown in Figure 2. The solubility of *n*-octyl, *n*-nonyl, *n*-decyl, and *n*-undecyl alcohols was so low that they were added to the manometer cups in the same manner as the hydrocarbons. The lower homologues were added as 0·01M aqueous solutions. Good oxygen uptakes were obtained with all the alchohols, except n-butanol which is poorly oxidized.

(e) *Oxidation of Saturated Aliphatic Carbonyl Compounds*

Four aldehydes only were available for testing, viz. acetaldehyde and its insoluble C_6, C_8, and C_{10} homologues. The latter three compounds were added to the cups in the same manner as the hydrocarbons. Acetaldehyde was readily oxidized without a lag period, giving a Q_{O_2} (N) value of 160, averaged over an experimental period of 3 hr. n-Hexyl aldehyde and n-octyl aldehyde both inhibited the endogenous respiration of the suspension. n-Decyl aldehyde is not oxidized consistently. Frequently inhibition of blank respiration occurred, yet net oxygen uptakes have been obtained giving a Q_{O_2} (N) value of 80 over a 3-hr incubation period. In contrast, acetone and methyl ethyl ketone are poorly oxidized, whereas excellent oxygen uptakes are obtained with methyl nonyl ketone. Using the same suspension whose

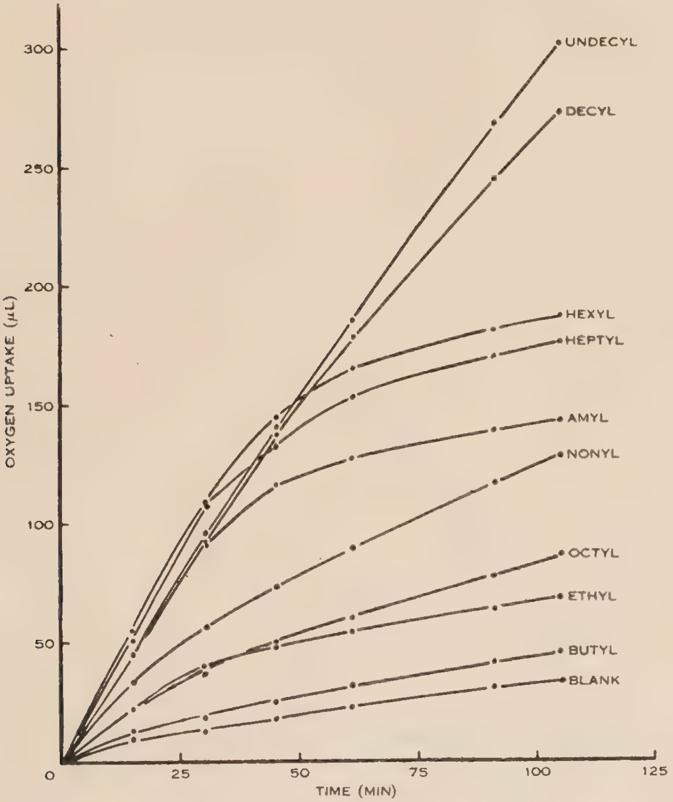


Fig. 2.—Oxidation of saturated monohydric alcohols. 0·1 ml of octyl, nonyl, decyl, and undecyl alcohols, and 0·1 ml of 0·01M solutions of the lower alcohols, 0·02M phosphate buffer pH 7·0, suspension 0·68 mg cell N, cup volume 2·5 ml, bath 25°C.

endogerous respiration is inhibited by *n*-decyl aldehyde, a Q_{O_2} (N) value of 445 was obtained for methyl nonyl ketone averaged over an incubation period of 2 hr.

(*f*) *Oxidation of Saturated Aliphatic Acids*

The rates of oxidation of the normal saturated monocarboxylic acids from formic to capric by the suspension are shown in Figure 3. Fatty acids, either with odd or even numbers of carbon atoms, are oxidized at the same rate without a lag period. For a given acid, the total oxygen uptake varies with the suspension, although good correlation is obtained between duplicate cups. In all cases, oxygen consumption was less than required for complete oxidation.

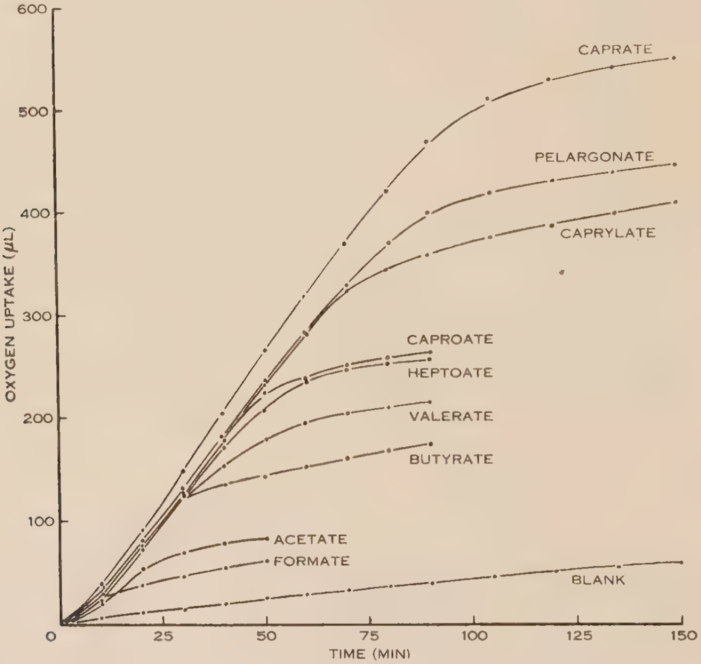


Fig. 3.—Oxidation of saturated monocarboxylic acids. 0·2 ml of 0·01M acid (sodium salt), 0·03M phosphate buffer pH 7·0, suspension 0·41 mg cell N, cup volume 2·5 ml, bath 25°C.

(*g*) *Co-oxidation of* n-*Decane with its Derivatives*

n-Decane was co-oxidized with *n*-decyl alcohol, *n*-decyl aldehyde, and *n*-decylic acid. The results are shown in Figure 4. The rate of oxidation of *n*-decane and *n*-decylic acid as co-substrates equals that of the faster oxidized individual compound (*n*-decylic acid). This proceeds until depletion of the fatty acid when the co-oxidation rate decreases to that of *n*-decane alone.

The oxidation rate of *n*-decane plus *n*-decyl alcohol exceeds the rate of oxidation of the individual compounds but is far less than the sum of the individual rates. The co-oxidation rate equals the initial high rate of *n*-decane oxidation and maintains this rate after *n*-decane oxidation decreases. Co-oxidation of *n*-decane plus *n*-decyl aldehyde proceeds at a rate intermediate between the individual rates.

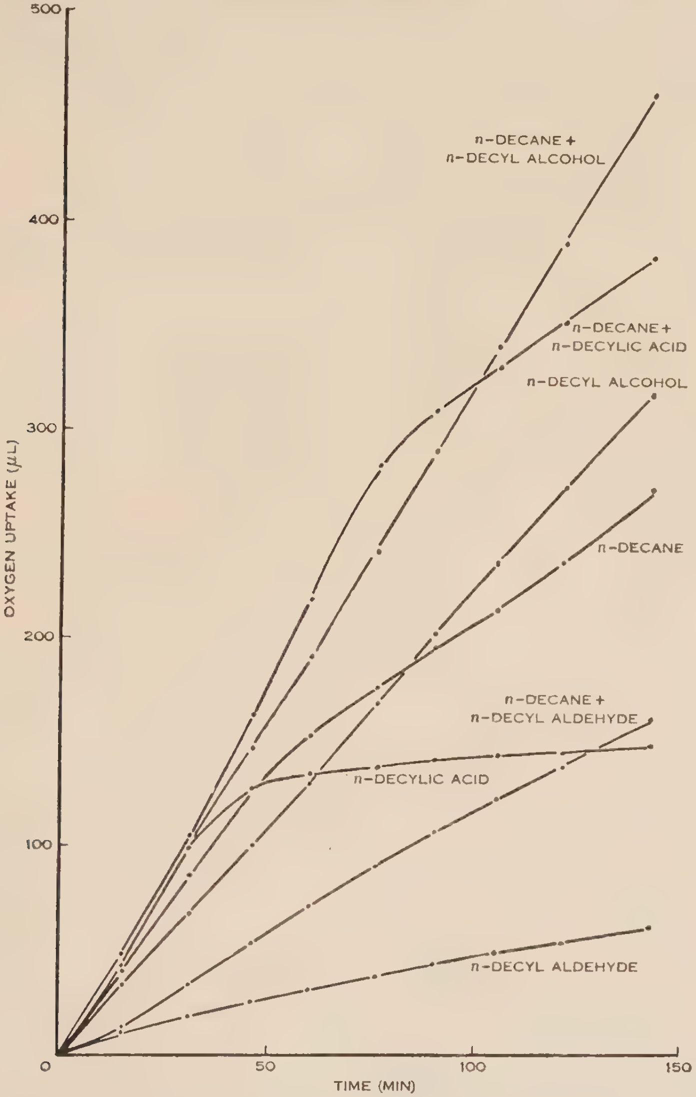


Fig. 4.—Co-oxidation of *n*-decane with its oxidative derivatives, corrected for endogenous respiration. 0·1 ml of each substrate except decylic acid which was added as a 0·01M solution, 0·03M phosphate buffer pH 7·0, suspension 0·27 mg cell N, cup volume 2·5 ml, bath 25°C.

(*h*) *The Effect of Inhibitors on* n-*Decane Oxidation*

The effects of various inhibitors on the oxidation of *n*-decane by the suspension are shown in Table 1. Experiments were carried out in cups containing 0·1 ml of hydrocarbon in 0·025M phosphate buffer at pH 7·0, except in the case of malonate where the pH was 6·0.

At pH 6·0, oxidation of 0·016M succinate was inhibited 50 per cent. by 0·024M malonate, but at this pH and concentration, malonate had no effect on *n*-decane oxidation. Fluoroacetate in concentrations of 0·02 and 0·004M inhibited the rate of oxidation of 0·004M sodium acetate by 66 and 38 per cent. respectively. Both concentrations inhibit *n*-decane oxidation 76 per cent. The initial fast rate of *n*-decane oxidation is unaffected by fluoroacetate which inhibits the secondary steady rate only.

In contrast, cyanide and azide appear to inhibit the initial rate only. The percentage inhibitions as tabulated for these compounds are functions of the period of oxidation. Cyanide (0·0008M) has no effect on *n*-decane oxidation. In the presence of 0·0016M cyanide, there is no preliminary high rate of oxidation. The oxygen uptake remains constant throughout and equal to the secondary rate in the absence of inhibitor. This is again observed in the presence of 0·0048M cyanide, but here there is a lag period before oxidation begins.

TABLE 1

THE EFFECT OF INHIBITORS ON *n*-DECANE OXIDATION

Inhibitor	Final Concentration	Cell N (mg/cup)	Percentage Inhibition
Malonate	0·024M	0·44	0
Fluoroacetate	0·004M	0·47	76
	0·02M	0·47	76
Cyanide	0·0008M	0·32	0
	0·0016M	0·32	50
	0·0048M	0·32	75
	0·008M	0·32	100
Azide	0·04M	0·32	0
	0·08M	0·32	72
	0·12M	0·32	90
Mercuric chloride	0·00001M	0·30	23
	0·0001M	0·30	100
Iodoacetate	0·0008M	0·30	88
Iodoacetate	0·0008M		
+cysteine	0·008M	0·30	0

The sulphydryl reagents inhibit both the initial and secondary oxidation rates. Cysteine reactivated both rates inhibited by iodoacetate.

(*i*) *Search for Products of* n-*Decane Oxidation*

n-Decane (0·1 ml) was added to a suspension of the organisms in a 100-ml conical flask and shaken at 25°C for varying times, after which the cup contents were brought to pH 1 with 5N H_2SO_4. An equivalent amount of suspension without the hydrocarbon was treated in the same manner.

(i) *Volatile Acids.*—The acidified flask contents were steam distilled and the distillate collected in standardized sodium hydroxide. Excess alkali was back-titrated with standard HCl using phenolphthalein as indicator. In three separate experiments, the control suspensions contained 1·74, 4·42, and 0·61 μ-equiv. of volatile acid per mg cell nitrogen respectively. However, no increase in volatile acid occurs during oxidation of *n*-decane.

Table 2

NON-VOLATILE ACID CONTENT OF SUSPENSIONS OXIDIZING *n*-DECANE

Suspension No.	Cell N (mg/cup)	Incubation Period (hr)	Non-volatile Acid (μ-equiv.)			
			Experimental		Blank	
			Acid 1	Acid 2	Acid 1	Acid 2
1	13·4	3	2·20	1·82	3·01	1·76
2	4·4	4	1·54	0·92	1·76	1·04
3	19·5	18	1·69	3·20	1·04	0·46

(ii) *Non-volatile Acids.*—Only two acids were discovered in the bacterial extracts but these were not positively identified. One acid ran identically on column and paper chromatograms with glutaric acid, the other with lactic acid, but no further identification was made. The results of several determinations are tabulated (Table 2). The corresponding peaks from the experimental and blank columns ran identically as single spots on two-dimensional paper chromatograms. The first and second column peaks respectively gave R_F values of 0·27 and 0·54 in the alkaline solvent and 0·86 and 0·75 in the acidic solvent.

IV. Discussion

Although none of the described species has morphological and biochemical properties completely identical to those of the organism studied, it is a member of the *Corynebacterium* genus (Jensen 1934; Bergey 1948). The organism belongs to the large sub-group which includes the saprophytic, soil-inhabiting diphtheroids, the properties of which are reviewed by Jensen (1952). Conn and Dimmick (1947) have suggested that many members of this group be classified in a separate genus, *Arthrobacter*, the type species of which is *A. globiforme*, a typical soil organism. This genus is characterized by marked changes in cell morphology during its life-cycle, ability to utilize simple inorganic sources of nitrogen, weak fermentative ability, and a tendency to be Gram-negative in young cultures but changing to Gram-positive in older cells. The bacterium studied possesses all of these properties, except that it is Gram-positive even in young cultures.

The ability to oxidize hydrocarbons is common to many groups of bacteria but it has not been shown to be a property widespread in the genus *Corynebacterium*. In fact Haag (1927) used the inability of corynebacteria to utilize paraffin as a means of differentiation of this class from the mycobacteria. Later, Bushnell and Haas (1941) showed that *C. simplex* and several unidentified and undescribed species of corynebacteria could grow in mineral media to which solid paraffin had been added. Only *C. simplex* utilized kerosene as a sole source of carbon. The bacterium under investigation differs from *C. simplex* in a number of properties and appears to be another member, previously undescribed, of this group, capable of actively metabolizing aliphatic hydrocarbons.

Very little positive information is available on the position of attack and mechanism of biological oxidation of aliphatic hydrocarbons. The lack of polar and ionic groups in the hydrocarbon molecule suggests that the first step in the oxidation is one of activation, due perhaps to the formation of free radicles.

Leach (1954) has recently reviewed the theories of mechanism of enzymatic oxido-reduction. Non-enzymatic autoxidation of hydrocarbons (RH) is initiated by the formation of the free radicle $R^{\cdot}$ which combines with oxygen to form the peroxide $RO_2^{\cdot}$. Further reaction of the peroxide with another hydrocarbon molecule results in the hydroperoxide and regeneration of a second $R^{\cdot}$ radicle, which repeats the process, thus

$$RO_2^{\cdot}+RH \rightarrow RO_2H+R^{\cdot}.$$

Thiyl radicles may be important in the initial activation. Bickel and Kooijman (1952) have shown that reactions of the type

$$RH+R_1S^{\cdot} \rightarrow R^{\cdot}+R_1SH$$

may be reversible at room temperatures and suggest their possible role in biological oxidations. The importance of the sulphydryl group in its association with enzymes responsible for oxido-reductions has been pointed out by Barron (1951). We have shown that *n*-decane oxidation is markedly inhibited by sulphydryl reagents, iodoacetate, and mercuric chloride, but that normal oxidation is fully restored by the addition of cysteine.

From an analysis of the end products of *n*-decane autoxidation at 145°C, Benton and Wirth (1953) concluded that *n*-decane is primarily attacked equally well at carbon atoms 2, 3, 4, and 5, but that terminal oxidation is negligible. In contrast, Berhard *et al.* (1952) have isolated stearic and palmitic acids containing deuterium from the body fat of rats fed on deuterium-labelled *n*-octadecane, suggesting oxidation at the terminal carbon atom. Whether the discrepancy observed between the positions of primary attack of hydrocarbons, autoxidized or by enzymatically controlled reactions, reflects basically different mechanisms of oxidation is still in doubt.

If the hydroperoxide were formed from the biological oxidation of the aliphatic hydrocarbons, then peroxidatic activity could result in the formation of the corresponding fatty alcohol which in turn could be oxidized to the fatty acid. This may then be oxidized by β-oxidation to carbon dioxide and water.

R.Q. measurements suggest that the hydrocarbon is completely oxidized. The lack of specific inhibitors and the ready oxidation of possible intermediates prevent an accumulation of the latter. The inability to demonstrate volatile acid formation from *n*-decane oxidation may be explained on this basis.

The ready oxidation of methyl nonyl ketone contrasted with the poorer and inconsistent oxidation of *n*-decyl aldehyde, suggests that oxidation of the hydrocarbon may proceed at a position other than the terminal carbon atom. However, it is doubtful whether the possible intermediates, tested for their ability to be oxidized by the suspension, really exist as such when produced enzymatically from the hydrocarbon. More probably they exist as activated forms as is the case for the oxidation of the fatty acids themselves. The non-oxidation of a suspected intermediate may be misleading due to an inability to form the activated derivative from the parent compound, yet, nevertheless, may be well oxidized as the activated form produced from the hydrocarbon itself.

Chromatographic analysis has revealed the formation of at least two non-volatile acids from *n*-decane oxidation. However, the same two acids are formed during growth of the organisms in nutrient broth. Production from *n*-decane was only demonstrated by depletion of the endogenous substrates by long incubation. It seems likely that the two acids are members of the general degradative pathway employed by the organisms and are not produced specifically from *n*-decane oxidation. The acids are chromatographically identical with lactic and glutaric acids but further identification has not been attempted.

Co-oxidation of *n*-decane with its oxidative derivatives, shows that in no case does the co-oxidation rate equal the sum of the individual rates. This indicates a common metabolic pathway, or at least the involvement of common group-specific enzymes at some stage in the degradation of the substrates.

V. Acknowledgments

I would like to thank Dr. R. J. Swaby for helpful discussions and Miss Grace Banos for technical assistance. I am especially indebted to Mr. J. R. Harris for staining and photographing the bacterial preparations.

VI. References

Barron, E. S. G. (1951).—*Advances in Enzymology* **11**: 201.

Benton, J. L., and Wirth, M. M. (1953).—*Nature*: **171**: 269.

Bergey, D. H. (1948).—"Bergey's Manual of Determinative Bacteriology." 6th Ed. (Ed. R. S. Breed, E. G. D. Murray, and A. P. Hitchens.) (The Williams and Wilkins Co.: Baltimore.)

Berhard, K., Gloor, U., and Scheitlin, E. (1952).—*Helv. Chim. Acta* **35**: 1908.

Bickel, A. F., and Kooijman, E. C. (1952).—*Nature* **170**: 211.

Bushnell, L. D., and Haas, H. I. (1941).—*J. Bact.* **41**: 653.

Clarke, P. H., and Cowan, S. T. (1952).—*J. Gen. Microbiol.* **6**: 187.

Conn, H. J., and Dimmick, I. (1947).—*J. Bact.* **54**: 291.

Dyar, M. T. (1947).—*J. Bact.* **53**: 498.

Haag, F. E. (1927).—*Zbl. Bakt.* (2 Abt.) **71**: 1.

Jensen, H. L. (1934).—*Proc. Linn. Soc. N.S.W.* **59**: 19.

Jensen, H. L. (1952).—*Annu. Rev. Microbiol.* **6**: 77.

Johnson, F. H., Goodale, W. T., and Turkevitch, J. (1942).—*J. Cell. Comp. Physiol.* **19**: 163.

Klieneberger-Nobel, E. (1950).—*Quart. J. Micr. Sci.* **91**: 340.

Ladd, J. N., and Nossal, P. M. (1954).—*Aust. J. Exp. Biol. Med. Sci.* **32**: 523.

Leach, S. J. (1954).—*Advances in Enzymology* **15**: 1.

Saz, A. K. (1949).—*Arch. Biochem.* **22**: 195.

Umbreit, W. W., Burris, R. H., and Stauffer, J. F. (1949).—"Manometric Techniques and Tissue Metabolism." 2nd Ed. (Burgess Publ. Co.: Minneapolis.)

Webb, R. B. (1954).—*J. Bact.* **67**: 252.

Webley, D. M., and de Kock, P. C. (1952).—*Biochem. J.* **51**: 371.

Zobell, C. E. (1950).—*Advances in Enzymology* **10**: 442.

Explanation of Plates 1 and 2

All preparations are coverslip impressions of the growth of *Corynebacterium* sp. on nutrient agar. Magnification × 1900.

Plate 1

Fig. 1.—8-Hr culture. Dyar cell-wall stain.

Fig. 2.—8-Hr culture. Webb cell-wall stain.

Fig. 3.—14-Hr culture. Giemsa stain showing occasional branching of the filaments.

Fig. 4.—24-Hr culture. Giemsa stain.

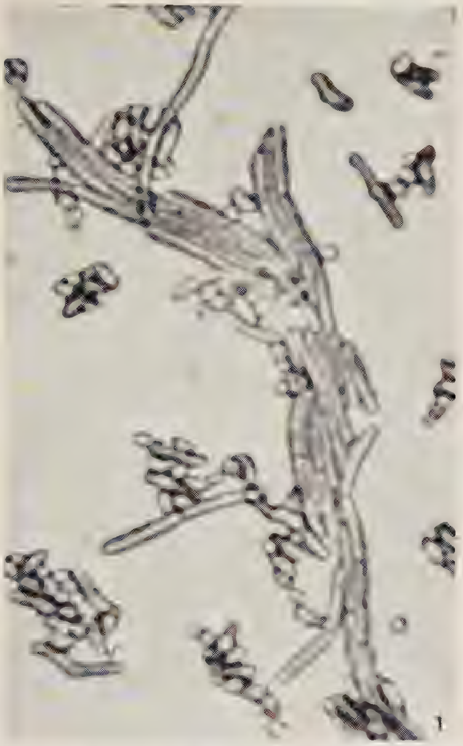
Plate 2

Fig. 1.—24-Hr culture. Webb cell-wall stain.

Fig. 2.—126-Hr culture. Dyar cell-wall stain.

Fig. 3.—126-Hr culture. Dyar cell-wall stain showing early stages of secondary growth.

THE OXIDATION OF HYDROCARBONS BY SOIL BACTERIA. I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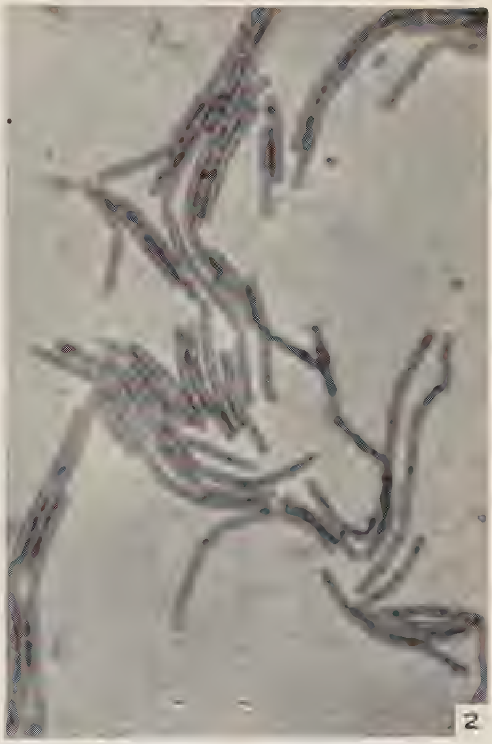
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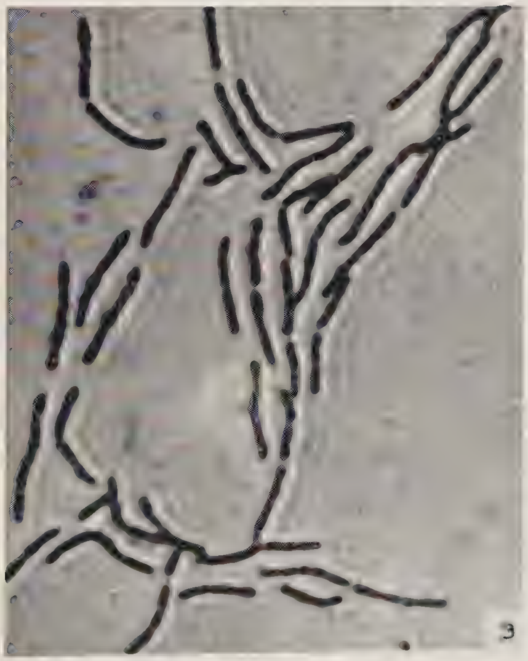
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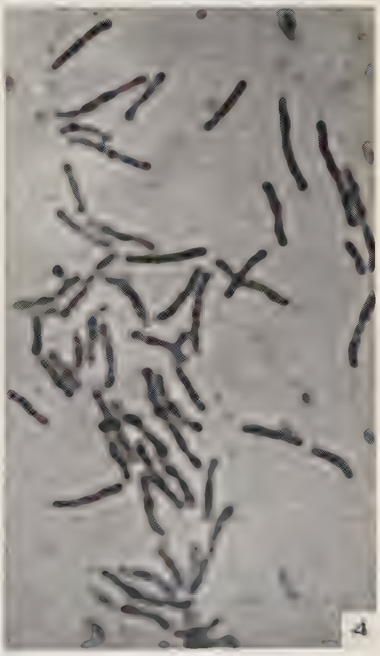
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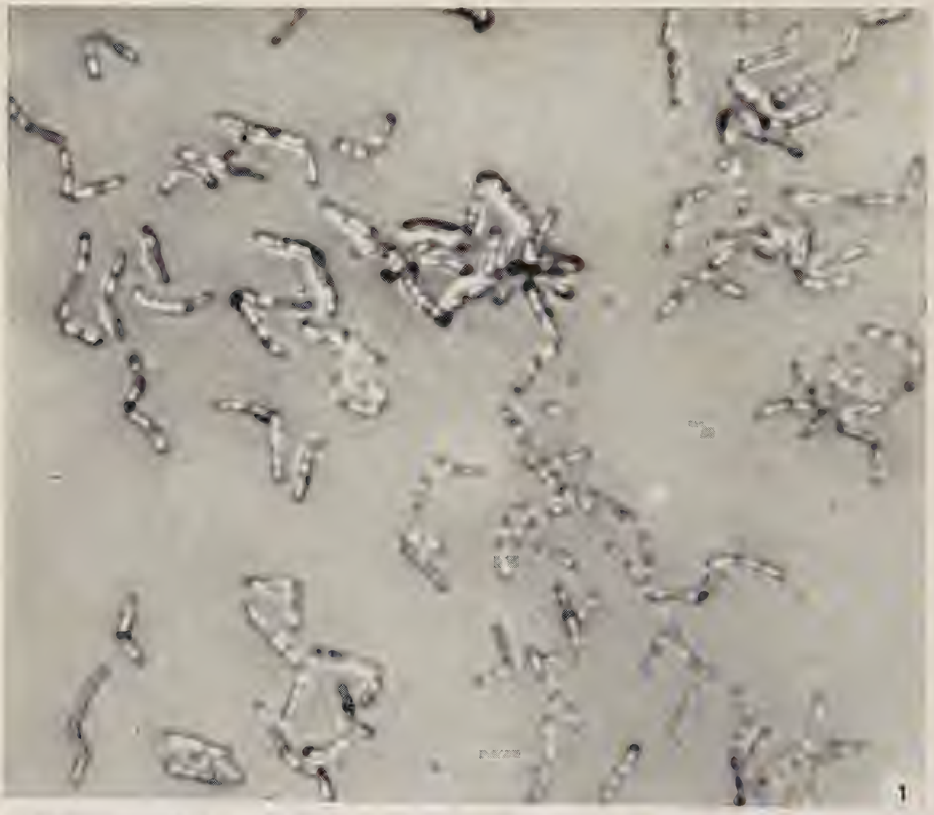
THE OXIDATION OF HYDROCARBONS BY SOIL BACTERIA. I



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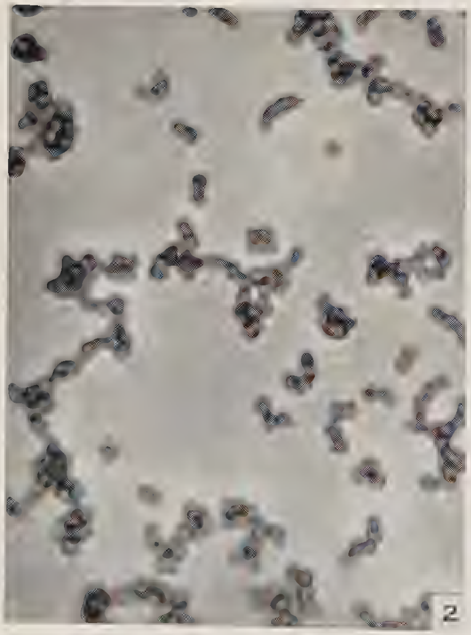

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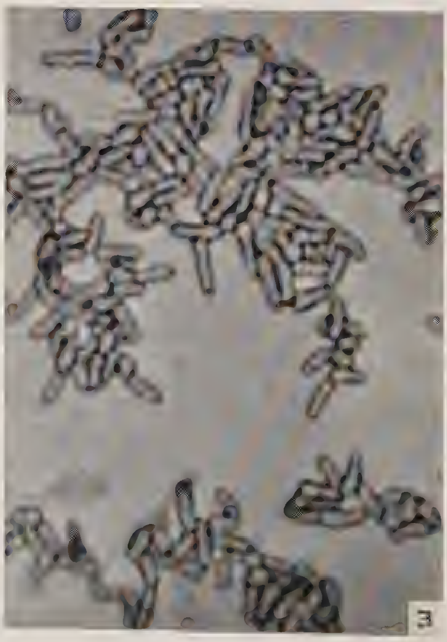

3

THE ROLE OF SERUM IN THE NUTRITION OF *ASTEROCOCCUS MYCOIDES*

By A. W. Rodwell*

[*Manuscript received September* 15, 1955]

Summary

For growth of *Asterococcus mycoides* in a complex liquid medium, serum could be replaced by balanced mixtures of a heat-stable protein fraction, cholesterol, and oleate, when a suitable osmotic pressure was provided. Oleate could be replaced by linoleate or by linolenate, and the protein fraction by bovine serum albumin or by starch. Both the protein fraction and cholesterol inhibited the rapid lysis brought about by low concentrations of oleate or other surface-active agents, anionic and non-ionic. It was concluded that serum provides a non-toxic source of oleate, probably as a molecular association with protein and cholesterol, but the evidence did not indicate whether or not cholesterol is also required as a nutrient.

I. Introduction

The causal organism of bovine pleuropneumonia, *Asterococcus mycoides*, like all strains of the pleuropneumonia group of microorganisms except those isolated from sewage (Laidlaw and Elford 1936) requires for growth in complex media factors which may be supplied by serum or ascitic fluid. Serum may be autoclaved with the medium without destruction of its growth-promoting properties (Priestley and White 1952), and it may be replaced by egg yolk (Nakamura, Futamura, and Watanuki 1926).

Preliminary work in this Laboratory indicated that lipids were involved in the growth-promoting activity of serum for *A. mycoides*. Serum extracted by the methods of Hardy and Gardiner (1910), McFarlane (1942), or of Sprince and Kupferberg (1947) would no longer support growth, but growth was restored in the presence of both the lipid extract emulsified in "Tween 80" as dispersing agent and of the lipid-extracted residue. When human plasma was fractionated by method 10 of Cohn *et al.* (1950), the activity was quantitatively recovered in the crude fractions III (o), IV (i), and V (i.e. the α- and β_1-lipoproteins and the albumin fraction). A lipoprotein prepared from horse serum (Macheboeuf and Rebeyrotte 1949) also had some activity in replacing serum.

Edward and Fitzgerald (1951) and Edward (1953) showed that both lipid and protein fractions of serum were required, and that the lipid fraction could be replaced by cholesterol. Serum could be replaced for a number of strains by a mixture of cholesterol and either starch or serum albumin. Growth was improved by the further addition of egg-yolk phospholipids. Cholesterol could be replaced by cholestanol or stigmasterol. Cholesterol hydrogen phthalate replaced cholesterol for some strains, but the oleate, stearate, and acetate esters were inactive for all strains. Smith and Morton (1951, 1952) described an electrophoretically pure, lipid-free, low molecular

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weight protein from serum which replaced serum for a number of strains of human origin.

These discordant findings have now been reconciled by a paper of Smith, Lecce, and Lynn (1954), in which the protein of Smith and Morton (1952) was shown to contain bound lipid, including cholesterol. After methanol extraction of both basal medium and protein, a requirement for cholesterol was found. Phospholipids in low concentrations increased the growth obtained in the presence of cholesterol and methanol-extracted protein. The inactivity of the oleate, acetate, and stearate esters was confirmed, but cholesteryl laurate was superior to cholesterol. Smith *et al.* (1954) postulated that the activity of the protein was due to its high cholesterol-binding capacity. It could be replaced by higher concentrations of lipid-extracted serum albumin or β-lactoglobulin.

Our investigations have been concerned with a strain of *A. mycoides*. This species was not studied by Smith *et al.* (1954). Although Edward (1953) found this species to be more fastidious in serum replacement experiments than other members of the genus, it grows readily in liquid media, and growth may therefore be evaluated quantitatively.

II. METHODS

(*a*) *Cultural Methods*

(i) *The Organism.*—Strain "V5" (Campbell 1938) was used.

(ii) *Basal Medium.*—The composition (per litre) of the basal medium was:

"Vitamin-free", acid-hydrolysed casein ≡	2·5 g casein
L-Cystine	5 mg
L-Tryptophan	5 mg
"Difco" yeast extract	10 g
Na_2HPO_4-KH_2PO_4 buffer, pH 7·4	0·04 mole.

Glucose (1 per cent. w/v) was added from a sterile 50 per cent. w/v solution after autoclaving the medium at 10 lb for 20 min. Tubes of medium having a final volume of 10 ml were capped with aluminium caps.

(iii) *Growth Conditions.*—The inoculum was prepared from a 3-4 day culture in BVF-OS medium (Turner, Campbell, and Dick 1935), containing between 10^{10} and 10^{11} viable particles per ml. The culture was centrifuged, the deposit was suspended in twice the original volume of saline buffered with 0·01M phosphate, and 1 drop was used to inoculate 10 ml of medium.

The tubes were incubated in air at 37°C for 3 or 4 days.

(iv) *Estimation of Growth.*—Two methods were used: optical density and titratable acidity. Optical density was measured using a filter transmitting maximally at 660 mμ, and with uninoculated medium as blank; titratable acidity was estimated by titrating both cultures and uninoculated medium in a stream of carbon dioxide-free nitrogen, to pH 8·0 as measured by a glass electrode. The two methods gave results in agreement.

(*b*) *Fractionation of Horse Serum*

(i) *Fraction A*.—Serum diluted with an equal volume of water was heated to 75°C, cooled, and its pH brought to 5·6 with N acetic acid; it was then heated to 80°C, cooled, and filtered on a Büchner funnel. Filtrate ≡ fraction A.

(ii) *Fraction* A_1.—Impermeate after dialysis of fraction A.

(iii) *Fraction* A_2.—Combined permeates from dialysis of fraction A, concentrated at reduced pressure, and neutralized.

(iv) *Fraction B*.—Fraction A (pH 6·4) was saturated with ammonium sulphate, the precipitate was dissolved in water (approximately one-twentieth of the original volume of serum), and the solution was dialysed.

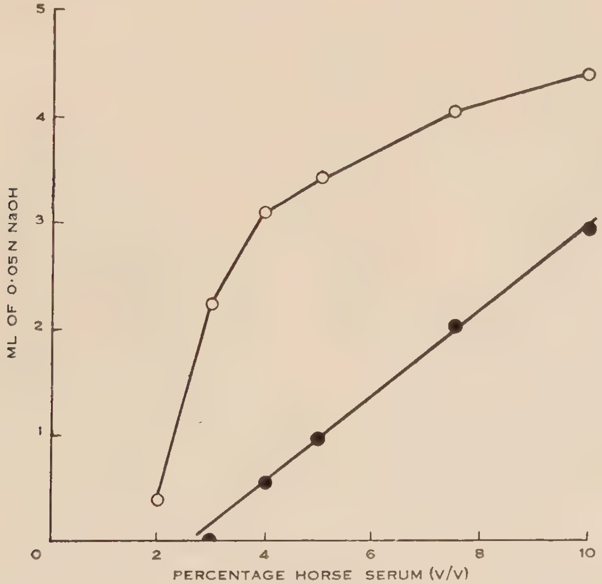

Fig. 1.—Effect of yeast extract concentration on serum requirement for growth of *A. mycoides*. ● 0·5 per cent. "Difco" yeast extract. ○ 1·0 per cent. "Difco" yeast extract.

(v) *Fraction C*.—Fraction B (protein content approximately 3 per cent. w/v) was chilled and added slowly to 10 volumes of acetone at −5°C, 1 ml of M acetate buffer pH 4·5 was added per litre of mixture, and the mixture was allowed to stand for 30 min at −5°C. The precipitate was washed successively with acetone, acetone-ether mixture, and ether, and extracted with ether for 6-10 hr in a Soxhlet extractor.

(*c*) *Lipids*

(i) *Reagents*.—Cholesterol was recrystallized twice from ethanol. Cholesteryl monosuccinate was prepared by the method of Cataline *et al.* (1944). Polyoxyalkalene sorbitan oleate ("Tween 80") was purified by ether extraction as described by Davis (1947), and kept as a 20 per cent. w/v solution at −80°C.

The samples of linoleic and linolenic acid were the *trans*-isomers. They were kept *in vacuo* at −80°C. The oleic acid was a commercial sample of redistilled oleic acid. It was used without further purification.

(ii) *Addition of Reagents to Medium.*—Unsaturated fatty acids were added to the medium either as soaps, or as ethanolic solutions. In the latter case the amount of solution added did not exceed 1 per cent. (v/v).

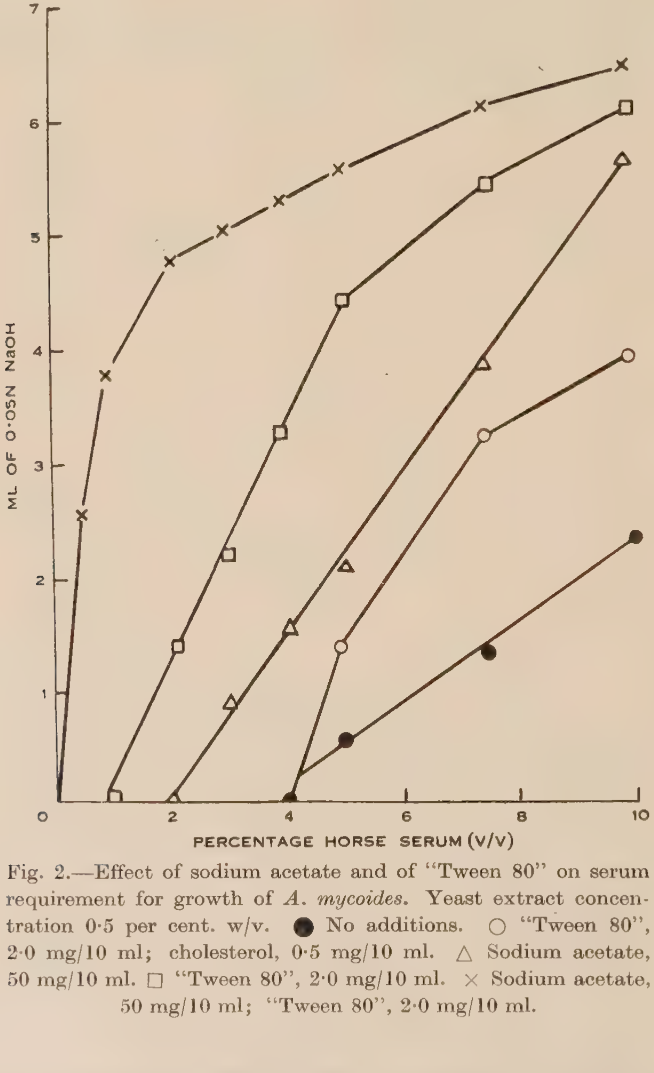

Fig. 2.—Effect of sodium acetate and of "Tween 80" on serum requirement for growth of *A. mycoides.* Yeast extract concentration 0·5 per cent. w/v. ● No additions. ○ "Tween 80", 2·0 mg/10 ml; cholesterol, 0·5 mg/10 ml. △ Sodium acetate, 50 mg/10 ml. □ "Tween 80", 2·0 mg/10 ml. × Sodium acetate, 50 mg/10 ml; "Tween 80", 2·0 mg/10 ml.

Cholesterol dispersion in "Tween 80" was prepared by adding 0·5 ml of an ethanolic solution at 60°C in a fine stream to 10 ml of "Tween 80" solution at 60°C. When cholesterol or its succinate was to be added without "Tween 80", a dispersion in water was prepared analogous to that in "Tween 80" solution. Faintly opalescent dispersions were obtained.

(iii) *Estimation of Phospholipid Content of Fraction C.*—Fraction C, (0·5 g) was refluxed four times with 20 ml portions of ethanol-ether (3:1) mixture. The combined extracts were evaporated to a small volume, and the phospholipids were precipitated with acetone containing a few drops of ethanolic solution of magnesium chloride. The acetone precipitate was dissolved in ether, and the total phosphorus of the solution was determined. The total phospholipid was calculated by multiplying the phospholipid phosphorus by the factor 25.

(iv) *Determination of Cholesterol Content of Fraction C.*—Cholesterol was determined in the supernatant, after acetone precipitation of the phospholipids, by the method of Sperry and Brand (1943).

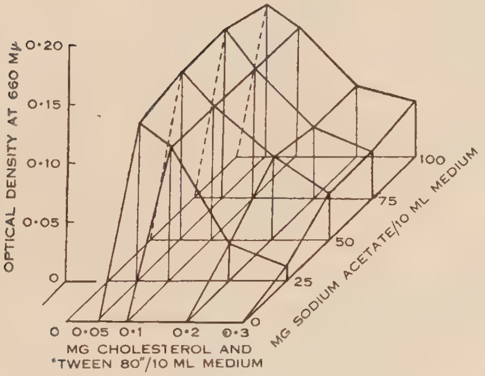

Fig. 3

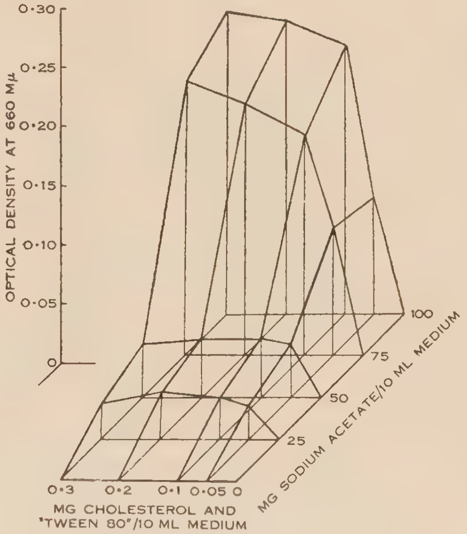

Fig. 4

Fig. 3.—Relationship between growth of *A. mycoides* and concentrations of cholesterol in "Tween 80" dispersion and concentrations of sodium acetate. Fraction C, 1·0 mg/10 ml medium.

Fig. 4.—Relationship between growth of *A. mycoides* and concentrations of cholesterol in "Tween 80" dispersion and concentrations of sodium acetate. Fraction C, 4·0 mg/10 ml medium.

(*d*) *Protein Estimation*

Protein was estimated by the biuret reaction as described by Weichselbaum (1946), with Armour's "Bovine Albumin Fraction V" as the standard.

III. EXPERIMENTAL

(*a*) *Growth Experiments*

(i) *Substances Sparing the Requirement for Serum.*—Acetate, "Tween 80", and yeast extract all had a pronounced effect on the quantitative requirement for serum. The effect of the yeast extract concentration is shown in Figure 1, and that of acetate and of "Tween 80" in Figure 2. Cholesterol, in the presence of whole serum, reversed the effect of "Tween 80" (Fig. 2). With ether-extracted serum, however, both cholesterol and "Tween 80" were essential for growth. A concentrate of α-lipoic acid did not spare the requirement for serum.

(ii) *Results of Horse Serum Fractionation.*—When serum was fractionated by the method set out in Section II (*b*), it was found that about half of the total activity of serum was recovered in fraction A, with approximately a four-fold purification. No growth was obtained when this fraction was autoclaved with the basal medium, although this did not cause any visible precipitate. It was necessary to add fraction A as a sterile Seitz filtrate after autoclaving the basal medium.

The activity of fraction A was lost after dialysis (fraction A_1), but was restored by the addition of the concentrated neutralized permeates (fraction A_2). Fraction A_2 contained the acetate used in the preparation of fraction A, and it was found that its activity could be totally accounted for by its acetate content.

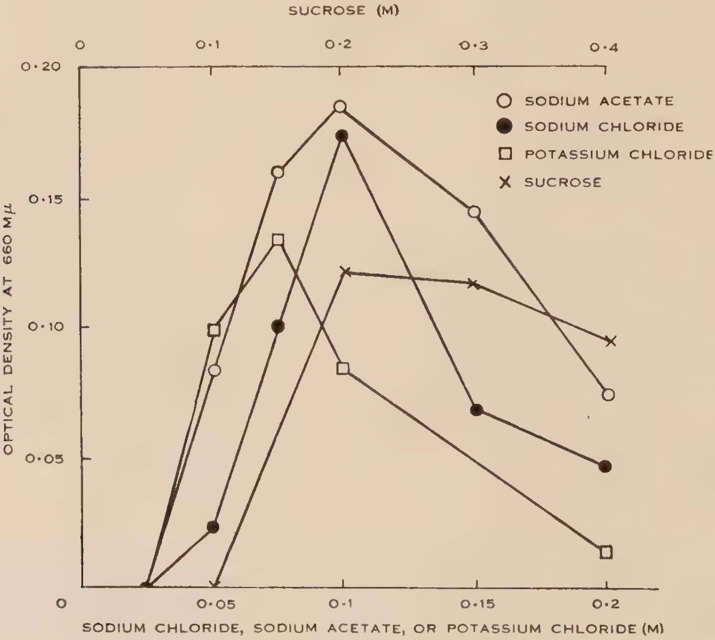

Fig. 5.—Replacement of sodium acetate by sodium chloride, potassium chloride, or sucrose for the growth of *A. mycoides*. Cholesterol, 0·5 μmole; sodium oleate, 0·15 μmole; fraction C, 5·0 mg/10 ml medium.

The activity of fraction A_1 was quantitatively recovered in the saturated ammonium sulphate precipitate (fraction B). In the presence of fraction C (i.e. fraction B after acetone-ether extraction) cholesterol, "Tween 80", and acetate were essential. The activity of fraction C, unlike that of fraction B, was not impaired by autoclaving. The optimum concentrations of cholesterol dispersion in "Tween 80", of fraction C, and of acetate were interrelated. This is illustrated in Figures 3 and 4.

(iii) *Specificity of Components.*—The specificity of the requirement for each of the components sodium acetate, "Tween 80", cholesterol, and the protein fraction was investigated.

(1) *Acetate.*—Sodium acetate could be replaced by approximately equimolar concentrations of other uni-univalent salts (e.g. sodium or potassium chloride), or by sucrose in twice these molar concentrations (Fig. 5).

(2) "*Tween* 80".—"Tween 80" was replaceable by oleate. In order to decrease the amount of growth in the absence of added oleate, the yeast extract used for the preparation of the basal medium was extracted with ether in a continuous extractor for 20 hr at pH 2·5. The growth responses to varying concentrations of oleate, cholesterol, and fraction C are illustrated in Figures 6 and 7. For 1 mg of fraction C per 10 ml (Fig. 6), the optimum oleate and cholesterol concentrations were 0·05 and 0·1 μmole per ml respectively. At this concentration of fraction C, an oleate concentration of 0·2 μmole per 10 ml completely suppressed growth at all cholesterol concentrations. For 5 mg of fraction C per 10 ml (Fig. 7), the optimum oleate and cholesterol concentrations were 0·1 and 0·5 μmole per 10 ml respectively, and growth was only slightly inhibited by oleate concentrations as high as 0·25 μmole per 10 ml. Oleate could be replaced by linoleate and linolenate (Fig. 8), although these acids became inhibitory at lower concentrations than oleate.

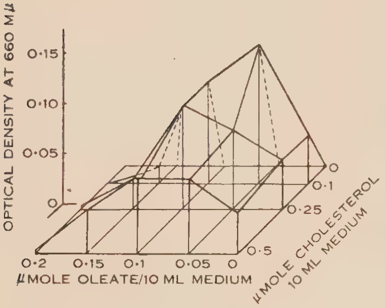

Fig. 6.

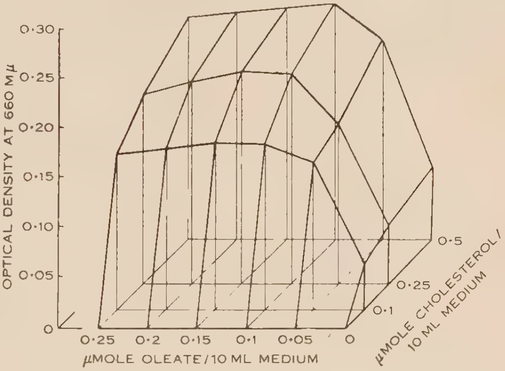

Fig. 7.

Fig. 6.—Relationship between growth of *A. mycoides* and concentrations of oleate and of cholesterol. Fraction C, 1·0 mg/10 ml medium; acetate 0·1M.

Fig. 7.—Relationship between growth of *A. mycoides* and concentrations of oleate and of cholesterol. Fraction C, 5·0 mg/10 ml; acetate 0·1M.

(3) *Cholesterol.*—It may be seen from Figures 6 and 7 that the optimum cholesterol concentration varied with the concentration of fraction C. The effect of varying the cholesterol concentration for a single concentration of fraction C and of oleate is illustrated in Figure 9. Cholesterol could not be replaced for growth by cholesteryl monosuccinate. No other esters or sterols were tested.

(4) *Fraction C.*—The activity of fraction C was destroyed by treatment with pepsin or pancreatin, but not by takadiastase; it was reduced by boiling for 10 min at pH 11, but it was unaffected by boiling for 10 min at pH 2·5. Paper electrophoresis in veronal buffer at pH 8·6 showed the presence of at least three protein components. One of the components, comprising approximately one-third of the protein, was insoluble at pH 4·8. The proteins soluble and insoluble at pH 4·8

were, however, of approximately equal activity. The activity and recovery of the protein insoluble at pH 4·8 remained unchanged after precipitation was repeated five times. It was evident that the activity of fraction C was not associated with a single protein component. Therefore the separation of the proteins in fraction C was not pursued.

Fraction C contained 3·8 per cent. (w/v) total phospholipid and 0·03 per cent. (w/v) of esterified cholesterol; its peptide content as measured by the biuret reaction, against the "Bovine Albumin Fraction V" standard, was 97 per cent. The presence of phospholipid in fraction C was not essential to its activity, since the residue after hot ethanol-ether extraction was still active in supporting growth. The optimum cholesterol concentration was decreased from 0·5 μmole per 10 ml to 0·1 μmole per 10 ml after ethanol-ether extraction, in a system containing 0·15 μmole oleate, and 5 mg of fraction C per 10 ml.

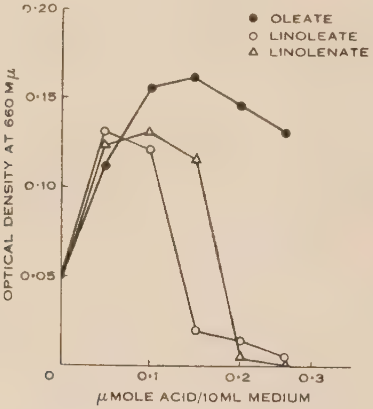

Fig. 8.

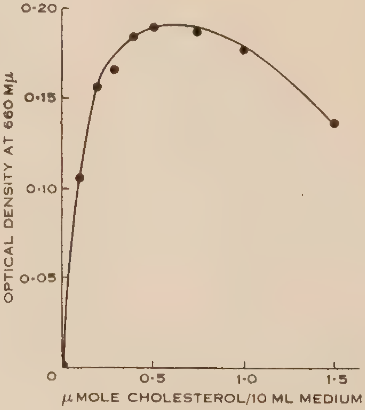

Fig. 9.

Fig. 8.—Replacement of oleate by linoleate and by linolenate for growth of *A. mycoides*. Fraction C, 5 mg/10 ml medium; cholesterol, 0·5 μmole/10 ml medium; acetate 0·1M.

Fig. 9.—Effect of cholesterol concentration on growth of *A. mycoides*. Oleate, 0·15 μmole; fraction C, 5 mg/10 ml medium; acetate 0·1M.

Fraction C was replaceable to a limited extent by 0·25 per cent. (w/v) of soluble starch, or by 0·25 per cent. (w/v) of "Bovine Serum Albumin Fraction V" after treatment with ethanol and ether. The optimum cholesterol concentration was much lower, and the amount of growth was only about one-quarter of that obtained with fraction C.

(*b*) *Lysis of* A. mycoides

(i) *Effect of Tonicity of the Medium*.—When cells of *A. mycoides* were suspended in dilute buffer solution (0·01M phosphate buffer pH 7·4), the optical density of the suspension measured at 660 mμ was lower than that of the culture from which it had been prepared. The decrease in optical density varied for different cultures from 20 to 30 per cent., and was accompanied by the release of 260 mμ-absorbing material from the cells into the suspending medium. The decrease of optical density was prevented by suspending cells in isotonic buffered saline, or in Krebs-Ringer-phosphate solution. Removal of calcium ions by including "Versene" (ethylenediaminetetra-acetic acid) in the suspending medium had no effect on the stability of suspensions.

(ii) *Lysis by Surface-active Substances.*—In these experiments, sodium chloride (0·15M) in 0·01M phosphate buffer (pH 7·4) was used as the suspending medium. Cells of *A. mycoides* are known to be rapidly lysed by surface-active substances such as the saponins and bile salts. Since the decrease in optical density of suspensions in the presence of surface-active agents was proportional to the amount of 260 mμ-absorbing material released, optical density changes were used as a measure of lysis. The effect of a number of surface-active substances on the optical density of cell suspensions is shown in Table 1.

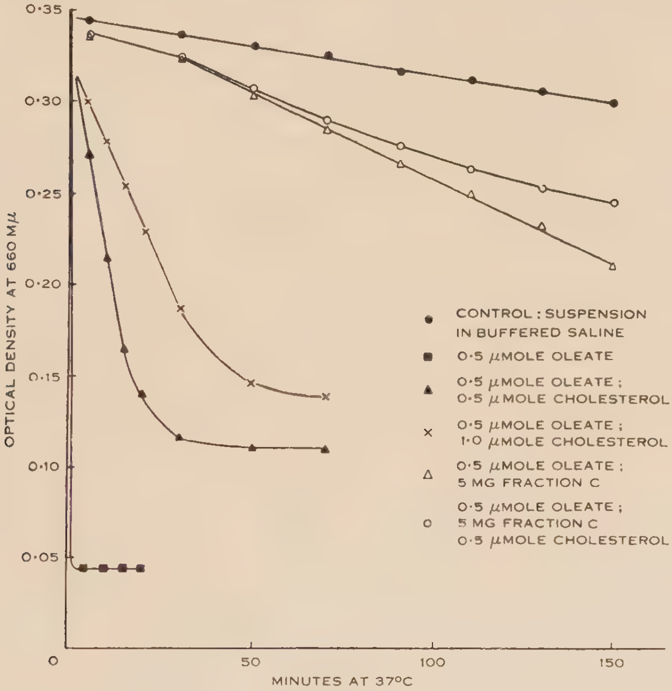

Fig. 10.—Inhibition of oleate lysis of *A. mycoides* by cholesterol and by fraction C. Mixtures of oleate and inhibitors incubated at 37°C for 15 min before system completed by addition of cells. Additions per 10 ml medium indicated in legend on figure.

(iii) *Absence of Lysis by Lecithinase C.*—Since cells of *A. mycoides* were susceptible to lysis by surface-active haemolytic agents, it was of interest to see whether they were susceptible to lysis by lecithinase C. It may be seen from Table 1 that *Clostridium welchii* lecithinase C did not cause lysis of cells of *A. mycoides*.

(iv) *Absence of Lysis by* Cl. welchii *θ-Haemolysin.*—The —SH-dependent *Cl. welchii* θ-haemolysin did not lyse cells of *A. mycoides* (Table 1).

(v) *Inhibition of Lysis Caused by Oleate.*—Cholesterol and fraction C both inhibited lysis by oleate. Both inhibitors together were more effective than either alone. This is illustrated in Figure 10. Approximately twice the molar concentration of cholesteryl monosuccinate as of the unesterified sterol was required to reverse the effect of the same amount of oleate.

TABLE 1

LYSIS OF CELLS OF A. MYCOIDES BY SURFACE-ACTIVE SUBSTANCES

Cultures in BVF-OS centrifuged at 18,000 *g* for 10 min, deposit washed once, and resuspended to a density of approximately 2 × 10^{10} cells per ml. Optical density of suspensions plotted against period of incubation at 37°C

Suspending Media	Compound	Nature	Concentration (μg/ml)	Incubation Time (min)	Decrease in Optical Density (%)
NaCl, 0·154M; phosphate buffer, 0·01M, pH 7·4	Oleic acid	Anionic	5	10	12
			10	10	80
,, ,,	Sodium lauryl sulphate	Anionic	30	10	8
			60	10	92
,, ,,	Sodium deoxycholate	Anionic	200	10	11
			300	10	85
,, ,,	"Tween 80"	Non-ionic	200	10	34
			400	10	64
,, ,,	Saponin	Non-ionic	20	10	19
			50	10	73
NaCl, 0·154M; tris (hydroxymethyl)-aminomethane-HCl buffer, pH 7·4; 0·005M $CaCl_2$	*Cl. welchii* α-toxin	Lecithinase C	90 HU*/ml	30	0
NaCl, 0·154M; phosphate buffer, 0·01M; sodium thioglycollate, 0·1% w/v	*Cl. welchii* θ-toxin	Oxygen-labile haemolysin	333 HU*/ml	30	0

* Haemolytic unit-amount of toxin liberating 50% of haemoglobin from sheep red cell suspension containing 7·5 mg haemoglobin per ml in 30 min at 37°C.

IV. DISCUSSION

The results reported in this paper are in general agreement with those of Edward and Fitzgerald (1951) and of Smith *et al.* (1954) but showed a requirement by *A. mycoides* for an unsaturated fatty acid in addition to cholesterol and a protein fraction. The experiments also showed that the optimum concentrations of these three factors were interrelated.

Cells of *A. mycoides* were extremely sensitive to lysis by surface-active agents, particularly by oleate. In this respect they resembled erythrocytes, but differed in their complete resistance to the lecithinase (α-toxin) and the —SH-dependent θ-toxin of *Cl. welchii*. Fraction C and cholesterol inhibited lysis by oleate, and a mixture of both was more effective than either inhibitor alone.

These results are explained most simply by assuming that a molecular association is formed amongst protein, cholesterol, and fatty acid, and that this provides a non-toxic source of the acid. Cholesterol may also be required as a nutrient, as postulated by Edward and Fitzgerald (1951) and by Smith *et al.* (1954).

Both Edward and Fitzgerald (1951) and Smith *et al.* (1954) found that the addition of phospholipids increased the growth obtained in the presence of cholesterol and protein. Phospholipids are known to reverse the inhibitory effects of unsaturated fatty acids on the growth of *Lactobacillus casei* (Kodicek 1949), and to enhance the antihaemolytic activity of cholesterol (Lee and Tsai 1942; Ponder 1946).

The lipid requirements can be finally assessed only when the complete nutritional requirements are known. Attempts to replace yeast extract by known growth factors have so far been unsuccessful. Yeast extract and other natural materials have been shown to contain substances inhibiting the growth of the L form of *Proteus* (Medill and O'Kane 1954).

When serum was replaced by an unsaturated fatty acid, cholesterol, and fraction C, the importance of the tonicity of the medium became apparent. This recalls the finding of Koser, Breslove, and Dorfman (1942) that a suitable salt concentration is necessary for the growth of strains of the genus *Brucella*. When cells of *A. mycoides* were suspended in dilute buffer solution, approximately 30 per cent. of the total amount of 260 mμ-absorbing material was released into the medium. Osmotic effects on cells of *A. mycoides* have been noted by Freundt (1952), who showed that the pH of the culture medium in which the cells were grown influenced their osmotic resistance. If unsaturated fatty acids are involved in membrane permeability (Kodicek 1949), it might be expected that they would also effect the sensitivity of cells to osmotic effects. The lack of a rigid cell wall, and the sensitivity to osmotic effects and to lysis by surface-active substances, are probably the most outstanding features distinguishing the pleuropneumonia group of organisms, and perhaps also the L forms, from other forms of bacteria.

V. Acknowledgments

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VI. References

Campbell, A. D. (1938).—*J. Coun. Sci. Industr. Res. Aust.* **11**: 119.

Cataline, E. L., Worrell, L., Jeffries, S. F., and Aronson, S. A. (1944).—*J. Amer. Pharm. Soc.* **33**: 107.

Cohn, E. J., et al. (1950).—*J. Amer. Chem. Soc.* **72**: 465.

Davis, B. D. (1947).—*Arch. Biochem.* **15**: 359.

Edward, D. G. ff. (1953).—*J. Gen. Microbiol.* **8**: 256.

Edward, D. G. ff., and Fitzgerald, W. A. (1951).—*J. Gen. Microbiol.* **5**: 576.

FREUNDT, E. A. (1952).—*Acta Path. Microbiol. Scand.* **31**: 561.

HARDY, W. B., and GARDINER, S. (1910).—*J. Physiol.* **40**: lxviii.

KODICEK, E. (1949).—*Symp. Soc. Exp. Biol.* **3**: 217.

KOSER, S. A., BRESLOVE, B. B., and DORFMAN, A. (1942).—*J. Infect. Dis.* **69**: 114.

LAIDLAW, P. P., and ELFORD, W. J. (1936).—*Proc. Roy. Soc.* B **120**: 292.

LEE, J. S., and TSAI, C. (1942).—*Quart. J. Exp. Physiol.* **31**: 281.

MACHEBOEUF, M., and REBEYROTTE, P. (1949).—Faraday Soc. Discussion No. 6, p. 62.

McFARLANE, A. S. (1942).—*Nature* **149**: 439.

MEDILL, M. A., and O'KANE, D. J. (1954).—*J. Bact.* **68**: 530.

NAKAMURA, N., FUTAMURA, H., and WATANUKI, T. (1926).—*J. Jap. Soc. Vet. Sci.* **5**: 194.

PONDER, E. (1946).—*J. Gen. Physiol.* **29**: 1.

PRIESTLEY, F. W., and WHITE, R. W. (1952).—*Vet. Rec.* **64**: 259.

SMITH, P. F., and MORTON, H. E. (1951).—*J. Bact.* **61**: 395.

SMITH, P. F., and MORTON, H. E. (1952).—*Arch. Biochem. Biophys.* **38**: 23.

SMITH, P. F., LECCE, J. G., and LYNN, R. J. (1954).—*J. Bact.* **68**: 627.

SPERRY, W. M., and BRAND, FLORENCE C. (1943).—*J. Biol. Chem.* **150**: 315.

SPRINCE, H., and KUPFERBERG, A. B. (1947).—*J. Bact.* **53**: 441.

TURNER, A. W., CAMPBELL, A. D., and DICK, A. T. (1935).—*Aust. Vet. J.* **11**: 63.

WEICHSELBAUM, T. E. (1946).—*Amer. J. Clin. Path.* **16**: 40.

THE METABOLISM OF ARGININE IN *SERRATIA MARCESCENS*

I. PATHWAYS OF SYNTHESIS AND DEGRADATION

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Summary

Serratia marcescens has no ornithine cycle, the enzyme arginase being absent. Arginine is not dissimilated in the manner of the streptococci and staphylococci as arginine desmidase is lacking.

Citrulline is synthesized from ornithine and carbamyl phosphate in the presence of acetone-dried cells. Evidence is given that argininosuccinic acid is an intermediate in the formation of arginine in *S. marcescens*.

The arsenolysis and phosphorolysis of citrulline, and the enzymic breakdown of carbamyl phosphate have been studied. The latter reaction proceeds either by hydrolysis or by the transfer of phosphate to adenosine diphosphate (ADP). The reaction sequence in which citrulline is converted to ornithine is discussed.

I. Introduction

The metabolism of arginine and related compounds in animals and microorganisms has been reviewed by Ratner (1954). A review by Slade (1954) deals with bacterial systems.

Grisolia and Cohen (1953) investigated the catalytic activity of various glutamic acid derivatives having an *N*-carbonyl group in the conversion of ornithine, carbon dioxide, and ammonia to citrulline. Studies by Grisolia and Cohen (1952) indicated that the role of these catalysts was in the production of a phosphorylated intermediate, formed in the presence of adenosine triphosphate (ATP), carbon dioxide, and ammonia.

Ratner and Petrach (1951) found that argininosuccinic acid is an intermediate in the conversion of citrulline to arginine in liver. The reaction required ATP and aspartic acid. Davison and Elliott (1952) presented chromatographic evidence for the formation of argininosuccinic acid from arginine and fumaric acid in extracts from animal and plant tissues and microorganisms. Oginsky and Gehrig (1952*a*) reported the properties of arginine desmidase from *Streptococcus faecalis*. No evidence could be obtained for the participation of inorganic phosphate, ATP, oxygen, or fumarate. The same authors (1952*b*, 1953), and also Knivett (1952, 1954*a*, 1954*b*, 1954*c*), Korzenovsky and Werkman (1953, 1954), and Slade, Doughty, and Slamp (1954) have shown that the reactions occurring in *Strep. faecalis*, *Strep. lactis*, and *Pseudomonas* are:

$$\text{arginine}+H_2O \rightarrow \text{citrulline}+NH_3 \quad \ldots\ldots\ldots\ldots(1)$$

$$\text{citrulline}+\text{ADP}+H_3PO_4 \rightarrow \text{ornithine}+CO_2+NH_3+\text{ATP} \quad \ldots(2)$$

$$\text{citrulline}+H_2O \xrightarrow{\text{arsenate}} \text{ornithine}+CO_2+NH_3 \quad \ldots\ldots(3)$$

$$\text{citrulline}+H_2O \xrightarrow{\text{phosphate}} \text{ornithine}+CO_2+NH_3 \quad \ldots\ldots(4)$$

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Reaction (1) is inhibited by magnesium and by arsenate. Magnesium is required for reactions (2) and (4) but not for reaction (3). Arsenolysis (reaction (3)) is competitively inhibited by phosphate and ornithine, while phosphorolysis (reaction (4)) is competitively inhibited by ornithine. One of the oxygen atoms released as CO_2 in phosphorolysis comes from phosphate (Stulberg and Boyer 1954).

Krebs, Eggleston, and Knivett (1955) have demonstrated reactions (3) and (4) but not reaction (2) in mammalian liver homogenates, and have suggested that those microorganisms which possess the arginine dihydrolase component (reactions (1) and (2)) have evolved this system to serve as an energy-yielding process if the substrate is present in the medium. Gale (1945) has suggested that the same system in homolactic fermenters may serve as a source of metabolic carbon dioxide for growth purposes.

Jones, Spector, and Lipmann (1955) found that incubation of extracts of *Strep. faecalis* or liver mitochondria with ammonium carbamate, CO_2, and ATP resulted in the formation of carbamyl phosphate (CAP). Transcarbamylation occurred on incubation of the extract, CAP, and ornithine to yield citrulline. The formation of CAP was reversible and required magnesium.

S. marcescens is an aerobe able to utilize ammonia and a simple carbon source for growth purposes. It is of interest therefore to compare arginine metabolism in this organism with that of mammalian tissue, the fungi, and those bacteria which possess the dihydrolase system.

II. Materials and Methods

(*a*) *Materials*

(i) *Buffers.*—Phosphate buffers were mixtures of 0·5M Na_2HPO_4 and KH_2PO_4. Arsenate and tris(hydroxymethyl)aminomethane buffers were prepared by mixing molar solutions with N HCl or N KOH and diluting.

(ii) N-*Acetyl Glutamic Acid.*—This substance was prepared according to the method of Bergmann and Zerfas (1928).

(iii) *Carbamyl Phosphate.*—Synthesis was by the method of Jones *et al.* (1955).

(iv) *Amino Acids.*—Commercial preparations of the L-isomers were used.

(*b*) *Methods*

(i) *Cell Preparations.*—A laboratory strain of *S. marcescens* was grown in Bunting's (1946) glycerol-ammonium citrate-salts medium. An arginine-requiring mutant was obtained by irradiation of the prototroph with ultraviolet light, and was isolated by the method described by Lederberg and Tatum (1946). Acetone-dried cells were prepared by pouring a thick suspension into 20 volumes of acetone at −15°C and collecting by filtration after 6 min. Drying was completed *in vacuo* over conc. H_2SO_4, and the preparations stored at −15°C. Dialysis was against distilled water for 3–4 hr at 2°C.

(ii) *Urease and Arginase.*—Experiments for the detection of these enzymes were carried out under the conditions described by Srb and Horowitz (1944).

(iii) *Estimations*.—Urea was determined by the method of Krebs and Henseleit (1932), arginine by the method of Dubnoff (1941), citrulline by the modification of Archibald's method described by Knivett (1954*b*), and CAP by the method of Jones *et al.* (1955). In experiments in which citrulline was estimated, deproteinization was carried out with a final concentration of 10 per cent. (w/v) trichloroacetic acid.

(iv) *Paper Chromatography*.—To identify argininosuccinic acid, the incubated material was deproteinized with 2 vol. of ethanol and evaporated to dryness *in vacuo* over conc. H_2SO_4. The residues were then taken up in 20 per cent. (v/v) ethanol and spotted on Whatman No. 1 filter paper. The chromatograms were developed with phenol-water solvent (4:1) or butanol-acetic acid-water (4:1:1) for a period of 15 hr with ascending flow. To detect citrulline synthesis, and ornithine as a breakdown product of citrulline, the same procedure for deproteinization was adopted and the chromatograms run in butanol-acetic acid-water. Suitable markers were selected for each experiment, those used being ornithine, arginine, citrulline, glycocyamine, and glutamic and aspartic acids. After drying, the papers were sprayed with 0·2 per cent. (w/v) ninhydrin in ethanol and developed in the dark, either for 24 hr at room temperature, or for 15 min at 60-70°C.

To test for monosubstituted guanidyl derivatives, 0·04 per cent. (w/v) α-naphthol in ethanol, followed by 0·1 per cent. (v/v) bromine in 5 per cent. (w/v) NaOH was used (Eden, Harrison, and Linnane 1954). Carbamyl derivatives of amino acids were detected with 1 per cent. (w/v) *p*-dimethylaminobenzaldehyde in N HCl (Fowden 1951).

III. Results

(*a*) *Growth Characteristics of the Arginine-requiring Mutant*

The mutant was found to grow on arginine or citrulline but not on ornithine when these substances were added to the minimal medium (Bunting's). CAP and *N*-acetyl glutamic acid were inactive, as were other amino acids, uracil, glycocyamine, creatine, and urea. Growth in the presence of arginine was proportional to the concentration up to 1 μmole/ml of Bunting's medium. It was found that acetone-dried cells of the mutant were unable to carry out the arsenolysis of citrulline (Table 2). When a large inoculum was placed in minimal medium, growth was usually observed after a delayed period. Acetone-dried cells of the organism grown in this manner were found to have regained the ability to decompose citrulline in the presence of arsenate. When the mutant was streaked out on minimal-agar medium, a few colonies appeared after 4 days incubation. In none of many colonies tested was this growth due to back-mutation.

(*b*) *Urease and Arginase*

Bergey (1948) states that *S. marcescens* will not grow on urea, KCl, and glucose. As the organism is capable of synthesizing all of its nitrogenous requirements from ammonia and a simple carbon source, urease cannot be present except the organism be impermeable to urea. Urease could not be detected in whole cells of the parent strain. The lack of arginase activity was shown in experiments extending over a 20-hr period at pH 9·5 and 27°C, no accumulation of urea being detected when washed cells were incubated with arginine in an atmosphere of nitrogen. Arginine was recovered quantitatively. The permeability of whole cells to arginine was indicated by the formation of argininosuccinic acid (see Section III (*c*)).

(*c*) *The Formation of Argininosuccinic Acid*

When the arginine-requiring mutant was plated out on Bunting's agar medium and a very small amount of citrulline placed on the surface of the agar, a halo of growth appeared around the site of the addition after 14 hr incubation. When whole, washed cells or acetone-dried cells of the parent strain were incubated with arginine and fumarate, but not with arginine alone, paper chromatograms showed the presence of a second ninhydrin-reacting substance. With phenol-water as the mobile phase the R_F value of this substance, which appeared below arginine, was 0·24. Ratner, Petrach, and Rochovansky (1953) gave a value of 0·27 for pure argininosuccinic acid. The evidence is compatible with a pathway of conversion of citrulline to arginine proceeding via argininosuccinic acid.

Table 1

THE SYNTHESIS OF CITRULLINE

The complete system contained 25 mg acetone-dried cells of the parent strain, 500 μmoles tris(hydroxymethyl)aminomethane buffer pH 7·4, 20 μmoles ornithine, 10 μmoles CAP. Final vol. 3·0 ml; system incubated for 30 min at 30°C

System Components	Citrulline Synthesized
No enzyme	None
Complete (zero time)	None
Complete	10·1 μmoles
No ornithine	None
No CAP	None

(*d*) *Arginine Desmidase*

This enzyme hydrolyses arginine without the intermediate formation of argininosuccinic acid. Under conditions in which a rapid breakdown of arginine occurs in several other microorganisms and also under conditions which give rise to rapid phosphorolysis of citrulline in *S. marcescens*, no breakdown of arginine was detected with either whole or acetone-dried cells of the parent strain.

(*e*) *The Synthesis of Citrulline from Ornithine*

Attempts to demonstrate the aerobic synthesis of citrulline by whole washed cells of the parent strain in the presence of ornithine, ammonia, and bicarbonate with fumarate as the oxidizable substrate were unsuccessful. Additions of glutamic acid, *N*-acetyl glutamic acid, separately or together, and the growth of the cells in the presence of ornithine made no difference. However, when acetone-dried cells of the parent strain were incubated with ornithine and CAP, citrulline synthesis occurred (Table 1). The identity of citrulline was verified by paper chromatography.

(*f*) *The Arsenolysis and Phosphorolysis of Citrulline*

The arsenolysis and phosphorolysis of citrulline are carried out by many bacterial and animal tissue preparations. Phosphorolysis by bacteria which possess the arginine dihydrolase component is stimulated by the addition of adenosine phosphates. No such stimulation occurs in the liver homogenate reported by Krebs *et al.* (1955). Phosphorolysis and the effect of added adenosine phosphates using acetone-dried cells of the parent strain is shown in experiment A of Table 2. The rate of evolution of CO_2 was not enhanced by the presence of phosphate acceptors.

The arsenolysis of citrulline occurred with acetone-dried cells of the parent but not the mutant strain (experiment B, Table 2). The Michaelis constant for this reaction at 40°C was $2{\cdot}1 \times 10^{-2}$M. Krebs *et al.* (1955) gave a value of $1{\cdot}4 \times 10^{-2}$M for the liver enzyme under the same conditions of pH and temperature.

TABLE 2

THE ARSENOLYSIS AND PHOSPHOROLYSIS OF CITRULLINE

Expt. A—100 Mg dialysed acetone-dried cells, 500 μmoles phosphate buffer pH 6·1, 20 μmoles $MgSO_4$. Expt. B—50 Mg dialysed acetone-dried cells of the parent strain or arginine-requiring mutant, 500 μmoles arsenate buffer pH 6·7. Final vol. 3·3 ml; systems incubated in an atmosphere of N_2 at 40°C

Expt.	Additions to System	Free CO_2 Produced in 60 Min (μl)
A	None	3
	Citrulline, 160 μmoles	78
	Citrulline, 160 μmoles; AMP, 20 μmoles	77
	Citrulline, 160 μmoles; ADP, 20 μmoles	71
B	None	None
	Citrulline, 160 μmoles	154
	Citrulline (enzyme fraction from mutant), 160 μmoles	None

(*g*) *The Breakdown of Carbamyl Phosphate*

Jones *et al.* (1955) demonstrated the synthesis of citrulline from ornithine and CAP, and also the formation of ATP from CAP and ADP, the reactions being catalysed by extracts of *Strep. faecalis* and liver mitochondria The breakdown of CAP by *S. marcescens* has been studied using acetone-dried cells of the mutant strain dialysed against distilled water (Table 3). The experiments were carried out under conditions comparable with those used for the breakdown of citrulline.

The reaction involving CAP in the presence of acetone-dried cells and $MgSO_4$ was found to be a first order reaction, whilst that involving the addition of ADP followed second order kinetics in the initial stages. The velocity constant for the spontaneous decomposition of CAP at 40°C and in the presence of 0·15M phosphate buffer pH 6·1, was calculated from the measurement of CO_2 output as $3{\cdot}7 \times 10^{-5}$ sec^{-1}. The half-life is therefore 5·2 hr.

IV. Discussion

The evidence obtained indicates that the metabolism of arginine and its precursors in *S. marcescens* is directed towards synthesis. Arginine is not dissimilated under conditions in which *Strep. faecalis*, *Staphylococcus aureus*, etc. break down arginine to form ornithine, CO_2 and ammonia with the concomitant synthesis of ATP. The lack of arginase activity shows that there is no ornithine cycle comparable to that found in mammals and certain fungi. Argininosuccinic acid is formed from arginine and fumarate, and the growth of an arginine-requiring mutant on citrulline suggests that the compound is an intermediate in the citrulline to arginine conversion. No arginine desmidase, the enzyme hydrolysing arginine, could be detected.

Table 3

THE BREAKDOWN OF CARBAMYL PHOSPHATE

85 Mg dialysed acetone-dried cells of the arginine-requiring mutant, 500 μmoles phosphate buffer pH 6·1; 10 μmoles CAP. Final vol. 3·3 ml; systems incubated in an atmosphere of N_2 at 40°C

Enzyme Fraction	Additions	Free CO_2 (μl)	
		14 Min	30 Min
None	None	18	21
None	$MgSO_4$, 20 μmoles	18	21
Boiled enzyme	None	18	21
Boiled enzyme	$MgSO_4$, 20 μmoles	19	25
Enzyme	None	45	94
Enzyme	$MgSO_4$, 20 μmoles	52	123
Enzyme	ADP, 20 μmoles	45	102
Enzyme	$MgSO_4$, 20 μmoles; ADP, 20 μmoles	134	194
Enzyme	ATP, 20 μmoles	35	100
Enzyme	$MgSO_4$, 20 μmoles; ATP, 20 μmoles	59	138

Arsenolysis and phosphorolysis of citrulline by acetone-dried cells of *S. marcescens* has been demonstrated, the latter reaction being similar to that in liver extracts in that adenosine monophosphate (AMP) and ADP do not enhance the rate. Jones *et al.* (1955) demonstrated the synthesis of citrulline from ornithine and CAP, and phosphorylation of ADP by CAP in extracts of *Strep. faecalis* and liver mitochondria. The reaction between ADP and CAP yielded ATP, CO_2, and ammonia, and required magnesium. The synthesis of citrulline from CAP and ornithine has been demonstrated in *S. marcescens*. The breakdown of CAP under conditions in which phosphorolysis of citrulline occurs has been studied. CAP is broken down in the absence of an added phosphate acceptor, the reaction rate being increased by the addition of magnesium. In the presence of ADP and magnesium a very marked stimulation of CO_2 output occurred.

If the reactions involved in citrulline breakdown are formulated as

$$\text{citrulline}+\text{phosphate} \rightleftharpoons \text{ornithine}+\text{CAP} \quad \ldots\ldots(5)$$

$$\text{CAP}+\text{H}_2\text{O} \xrightarrow{\text{Mg}^{++}} \text{CO}_2+\text{NH}_3+\text{phosphate} \quad (6)$$

$$\text{CAP}+\text{ADP} \overset{\text{Mg}^{++}}{\rightleftharpoons} \text{CO}_2+\text{NH}_3+\text{ATP} \quad \ldots(7)$$

then the lack of stimulation of phosphorolysis by adenosine phosphates would be explained if reaction (**5**) is the rate-limiting reaction. The data of Jones *et al.* (1955) and the data for *S. marcescens* show that the equilibrium position for reaction (**5**) lies well to the left. Reaction (**6**) has been shown to be a first order reaction, and reaction (**7**) a second order reaction.

It is suggested that the differences between the mammalian system which splits citrulline, and the system in bacteria possessing the arginine dihydrolase component, may be explained in terms of the two reactions in which CAP is dissimilated. Preliminary experiments using cell-free extracts indicate that reactions (**6**) and (**7**) are catalysed by different enzymes.*

The reaction mechanism proposed by Krebs *et al.* (1955) for the citrulline-ornithine conversion, made prior to the identification of CAP as a phosphorylated intermediate, should now be written

$$E_1+\text{citrulline} \rightleftharpoons E_1\text{-citrulline} \overset{\text{phosphate}}{\rightleftharpoons} E_1\text{-ornithine}+\text{CAP} \quad \ldots(8)$$

$$E_1\text{-citrulline} \xrightarrow{\text{arsenate}} E_1\text{-ornithine}+\text{CO}_2+\text{NH}_3 \quad \ldots\ldots(9)$$

$$E_2+\text{CAP} \rightleftharpoons E_2\left\langle\begin{array}{l}\text{carbamyl} \rightleftharpoons \text{NH}_2\text{COOH}\\ \text{phosphoryl} \rightleftharpoons \text{ATP}\end{array}\right. \quad \ldots\ldots\ldots(10)$$

$$E_3+\text{CAP}+\text{H}_2\text{O} \rightarrow E_3+\text{CO}_2+\text{NH}_3+\text{phosphate} \quad \ldots\ldots(11)$$

where E_1, E_2, and E_3 are the enzymes catalysing the respective reactions.

The evidence presented in this paper is consistent with the hypothesis that the arginine-requiring mutant lacks the enzyme E_1. Reaction (**10**), in which the enzyme anhydride link is to phosphoryl, would account for the evidence of Stulberg and Boyer (1954) who demonstrated the incorporation of ^{18}O from labelled inorganic phosphate into CO_2 arising during the phosphorolysis of citrulline.

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* The enzyme catalysing reaction (7) has been obtained free of detectable phosphatase activity, and the stoichiometry of the reaction established.

VI. REFERENCES

BERGEY, D. H. (1948).—"Bergey's Manual of Determinative Bacteriology." 6th Ed. (Ed. R. S. Breed, E. G. D. Murray, and A. P. Hitchins.) (The Williams and Wilkins Co.: Baltimore.)

BERGMAN, M., and ZERFAS, L. (1928).—*Biochem. Z.* **203**: 280.

BUNTING, M. I. (1946).—*Cold Spr. Hbr. Symp. Quant. Biol.* **11**: 25.

DAVISON, D. C., and ELLIOTT, W. H. (1952).—*Nature* **169**: 313.

DUBNOFF, J. W. (1941).—*J. Biol. Chem.* **141**: 711.

EDEN, E., HARRISON, D. D., and LINNANE, A. W. (1954).—*Aust. J. Exp. Biol. Med. Sci.* **32**: 333.

FOWDEN, L. (1951).—*Biochem. J.* **48**: 327.

GALE, E. F. (1945).—*Brit. J. Exp. Path.* **26**: 225.

GRISOLIA, S., and COHEN, P. P. (1952).—*J. Biol. Chem.* **198**: 561.

GRISOLIA, S., and COHEN, P. P. (1953).—*J. Biol. Chem.* **204**: 753.

JONES, M. E., SPECTOR, L., and LIPMANN, F. (1955).—*J. Amer. Chem. Soc.* **77**: 819.

KNIVETT, V. A. (1952).—*Biochem. J.* **50**: xxx.

KNIVETT, V. A. (1954*a*).—*Biochem. J.* **56**: 602.

KNIVETT, V. A. (1954*b*).—*Biochem. J.* **56**: 606.

KNIVETT, V. A. (1954*c*).—*Biochem. J.* **58**: 480.

KORZENOVSKY, M., and WERKMAN, C. H. (1953).—*Arch. Biochem. Biophys.* **42**: 204.

KORZENOVSKY, M., and WERKMAN, C. H. (1954).—*Biochem. J.* **57**: 343.

KREBS, H. A., EGGLESTON, L. F., and KNIVETT, V. A. (1955).—*Biochem. J.* **59**: 185.

KREBS, H. A., and HENSELEIT, K. (1932).—*Hoppe-Seyl. Z.* **210**: 33.

LEDERBERG, J., and TATUM, E. L. (1946).—*J. Biol. Chem.* **165**: 381.

OGINSKY, E. L., and GEHRIG, R. H. (1952*a*).—*J. Biol. Chem.* **198**: 799.

OGINSKY, E. L., and GEHRIG, R. H. (1952*b*).—*J. Biol. Chem.* **198**: 791.

OGINSKY, E. L., and GEHRIG, R. H. (1953).—*J. Biol. Chem.* **204**: 721.

RATNER, S. (1954).—*Advances in Enzymology* **15**: 319.

RATNER, S., and PETRACH, B. (1951).—*J. Biol. Chem.* **191**: 693.

RATNER, S., PETRACH, B., and ROCHOVANSKY, O. (1953).—*J. Biol. Chem.* **204**: 94.

SLADE, H. D. (1954).—"Streptococcal Infections." (Columbia Univ. Press: New York.)

SLADE, H. D., DOUGHTY, C. C., and SLAMP, W. C. (1954).—*Arch. Biochem. Biophys.* **48**: 338.

SRB, A., and HOROWITZ, N. H. (1944).—*J. Biol. Chem.* **154**: 129.

STULBERG, M. P., and BOYER, P. D. (1954).—*J. Amer. Chem. Soc.* **76**: 5569.

THE RATE OF DIGESTION OF HUMAN BLOOD BY CERTAIN SPECIES OF MOSQUITOES

By A. K. O'Gower*

[*Manuscript received July* 25, 1955]

Summary

The rate of digestion of human blood was investigated at 4-hourly intervals by the precipitin and benzidine tests for the mosquito species *Aedes aegypti*, *A. concolor*, *A. notoscriptus*, *A. scutellaris*, and *Culex fatigans*. In an environment in which temperature and humidity were constant and in which there were equal periods of light and darkness, the rate of digestion ranged from 31 hr for *A. scutellaris* to 48 hr for *A. concolor*.

Increasing the proportion of darkness during the test period increased the rate of digestion in *A. notoscriptus*.

In experiments with *A. aegypti* and *A. scutellaris* the rate of digestion was similar in females 1 and 3 weeks old.

The benzidine test showed that haematin was defaecated for up to 90 hr after a blood meal.

I. Introduction

The "rate of digestion"† of blood by a female mosquito may affect its efficiency as a vector of diseases, since a female which digests its blood meal rapidly may feed at more frequent intervals than one which takes longer to digest its meal. It is of some value to know the rate of digestion for other purposes also; for example, the period of time during which the precipitin test can determine the origin of the host's blood is dependent upon the rate of digestion of the blood.

The precipitin test has been used previously to determine the arbitrary end-point of digestion of the blood serum proteins (reviewed by Bates 1949; West and Eligh 1952; Weitz and Buxton 1953) but these determinations were insufficiently critical to show finer differences in the rate of digestion by various species of mosquitoes. The benzidine test, however, has not been used previously in such studies and, although certain precautions are necessary in executing and interpreting it, the test was used in preference to others because it is so sensitive in detecting minute amounts of iron porphyrins.

The time required for the digestion of blood has been shown by Shlenova (1938) and West and Eligh (1952) to depend upon temperature and to a lesser extent upon humidity, but the effect of the period of illumination upon this process does not seem to have been studied. Therefore, the rate of digestion for *Aedes notoscriptus* was investigated under various conditions of light and darkness to determine whether this rate was affected by variation of these conditions.

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† The "rate of digestion" herein refers to the period of time required for blood meals of various mosquito species to reach such a stage of digestion that the serum proteins no longer precipitate their specific antibodies.

II. METHODS

The mosquitoes used were females of *A. aegypti*, *A. concolor*, *A. notoscriptus*, *A. scutellaris*, and *Culex fatigans* which had not fed previously. Those which fed on human blood were transferred to 4-in. gauze cages without food or water, and were kept in an incubator with a glass door. The temperature of the incubator was 27±1°C, and the saturation deficit was 2±1 mm Hg. Groups of the fed adults were killed at 4-hourly intervals, smeared whole onto filter papers (Eligh 1952), and kept dry in a refrigerator until the precipitin and benzidine tests could be applied to them.

TABLE 1

THE RATE OF DIGESTION OF HUMAN BLOOD BY VARIOUS SPECIES AS DETERMINED BY THE PRECIPITIN TEST

In this and subsequent tables, figures show the percentage of mosquitoes which gave positive reactions for either the precipitin test or the benzidine test, whilst the figures in brackets show the number of mosquitoes tested

Mosquito Species	Percentages and Numbers of Mosquitoes (in brackets) giving Positive Precipitin Tests								50% Rate of Digestion (hr)
	Time after Ingestion of a Blood Meal (hr)								
	24	28	32	36	40	44	48	52	
A. scutellaris	100(8)	100(21)	42(57)	0(15)	0(4)	0(11)	0(21)	0(7)	31
A. notoscriptus	100(20)	100(18)	100(59)	70(42)	28(57)	0(20)	0(15)	0(7)	36
A. aegypti		100(20)	100(25)	51(43)	0(17)	0(14)			38
C. fatigans			100(8)	100(15)	100(27)	60(30)	34(26)	0(15)	46
A. concolor					100(7)	100(12)	34(15)	0(6)	48

The influence of the ratio of light to darkness upon the rate of digestion of *A. notoscriptus* was determined as follows. After having been fed, the mosquitoes were divided into five lots, one lot being kept in each of the following conditions for 36 or 40 hr: constant darkness, constant light, light to darkness ratios of 2:1, 1:1, and 1:2. In the dark period, the glass door of the incubator was blacked out. In the light period, a 40-W incandescent tube was placed 9 in. in front of the glass door. At the end of the 36- or 40-hr period the mosquitoes were killed and smeared onto filter papers, which were then checked by the precipitin test for the presence of undigested serum proteins.

The process of digestion of blood by *C. fatigans* was observed at 12-hourly intervals and correlated with the rate of digestion which had been determined by the precipitin test.

In performing the precipitin tests, the filter paper smears were extracted with physiological saline, the extracts were run up small bore capillary tubes, and the specific antisera (prepared from laboratory animals after the method of Proom 1943) were run up the capillary tube beneath them. The details of this test were given in a previous paper (Lee, Clinton, and O'Gower 1954).

Blood smears and defaecated matter collected on the bottoms of the 4-in. gauze cages (after the digestion of the serum proteins), were checked for the presence of iron porphyrins by applying the benzidine test to them, after they had been heated in a steam oven for 10 min at 110°C. The benzidine test was checked for specificity in detecting the iron porphyrins in the mosquito abdomen by testing unfed, sugar-fed, and blood-fed adults of *A. scutellaris*. Only those mosquitoes which had fed on blood gave positive reactions.

III. RESULTS

At constant temperature and humidity and when the ratio of light to darkness was 1:1, there were differences in the rates of digestion of human blood for the five mosquito species tested (Table 1). By interpolation, the rates of digesion of 50 per cent. of the population varied from 31 hr for *A. scutellaris* to 48 hr for *A. concolor*.

From visual observations of *C. fatigans* at 12-hourly intervals, the progress of digestion was as follows: immediately after the ingestion of a blood meal the female mosquitoes were fully engorged with red blood; this turned black within 6 hr. At 12 hr, the abdomen was slightly more than three-quarters distended with blood. At 24 hr, the distended abdomen was about two-thirds filled with blood. After 36 hr. only the anterior half of the distended abdomen was filled with blood. At 48 hr, slightly less than half of the abdomen was distended with blood. Sixty hr after the meal, the abdomen was less than one-third distended with the breakdown products of digestion. During this period the distention of the abdomen gradually decreased, until at 60 hr it had the appearance of a gravid mosquito.

TABLE 2

THE INFLUENCE OF THE RATIO OF LIGHT TO DARKNESS UPON THE RATE OF DIGESTION OF HUMAN BLOOD BY A. NOTOSCRIPTUS

Time (hr)	Percentages and Numbers of Mosquitoes (in brackets) giving Positive Precipitin Tests				
	Constant Illumination	Light to Darkness Ratio 2:1	Light to Darkness Ratio 1:1	Light to Darkness Ratio 1:2	Constant Darkness
36	100(27)	100(30)	70(42)	54(48)	46(54)
40	40(50)	33(83)	28(57)	37(60)	0(25)

At constant humidity and temperature, and when the ratio of light to darkness varied in a given period (the critical digestion period in Table 1 (i.e. 36-40 hr)) from constant illumination to constant darkness, the rate of digestion of *A. notoscriptus* increased when the proportion of darkness in each period was increased (Table 2).

Positive reactions for the benzidine test were obtained up to 90 hr after ingestion, and, as defaecated matter collected on the bottom of the cages after the digestion of the blood meal also gave positive reactions, it was concluded that the haem of the haemoglobin was undigested and defaecated as a haematin-like substance.

IV. Discussion

The progress of digestion of blood meals by mosquitoes has been studied by several methods. These include the precipitin test used by Bull and King (1923), the histological method of Huff (1934), the observational studies of Shlenova (1938) and Hosking and MacInnes (1948), the crystallization method of Biocca (1950), and the studies on proteolytic digestion of blood by Fisk and Shambaugh (1952).

The benzidine test is non-specific, but it is extremely sensitive for the detection of iron porphyrins, of which haematin, a product of certain types of haemoglobin digestion, is one. From the results of this investigation it was apparent that iron porphyrins were present in the mosquito abdomen long after the serum proteins had been digested, and presumably also after the digestion of the globin moiety of haemoglobin. As defaecated matter also gave the same reaction, it was deduced that a haematin-like substance was defaecated rather than digested. This is in keeping with West and Eligh's finding (1952) with mosquitoes using the Meyer reduced phenolphthalein test. Wigglesworth (1943) obtained similar results when investigating the digestion of blood by *Rhodnius prolixus*.

There was no difference in the rate of digestion of 1- and 3-week-old adult mosquitoes; thus the use of a population in which the ages of the females varied had no effect upon the rates obtained.

Forty-eight hr after the ingestion of a blood meal by *C. fatigans* the precipitin test no longer gave a positive reaction, and, from visual observations, this time corresponded to the stage in digestion when the abdomen was slightly less than half distended, and it was then that the practical limitation of the precipitin test was reached in identifying the blood meal source.

It has been shown by previous authors that the rate of digestion of blood increases with rising temperature (Shlenova 1938; West and Eligh 1952) and with increasing humidity at low temperature (Shlenova 1938). The experiments recorded here show that it also increases as the ratio of dark to light increases for the species *A. notoscriptus*. However, as the general activity rhythms of all mosquito species would not be the same under similar conditions of illumination, it is not possible to generalize from this observation.

The rate of digestion of blood shows a wide range of specific variations which appear small when the measurements are made at 24-hr intervals (West and Eligh 1952). By using 4-hourly intervals between determinations, these differences become more obvious. Although Hovanitz (1947) showed that "the number of *Plasmodium gallinaceum* cysts" ingested by the adults of *A. aegypti* in a blood meal varied according to the size of the adult, such an explanation could hardly be given for these specific differences in the rates of digestion, since the intraspecific variation in the size of the adults was almost as great as the interspecific variation. Thus the different rates of digestion of human blood by the species of mosquitoes tested would seem to be due to specific differences in the digestive processes and not to specific differences in the size of the adults.

V. ACKNOWLEDGMENTS

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VI. REFERENCES

BATES, M. (1949).—"The Natural History of Mosquitoes." (The Macmillan Co.: New York.)

BIOCCA, E. (1950).—Le ricerche del "fenomono di gelificazione" e dei "gruppi cristallografici sanguigni d. Amantea" quali prove orientative per la identificazione del sangue ottenuto dallo stomaco delle zanzare. *Nuovi Ann. Igiene e Microbiol.* **1**: 10-23.

BULL, C. G., and KING, W. V. (1923).—The identification of the blood meal of mosquitoes by means of the precipitin test. *Amer. J. Hyg.* **3**: 491-513.

ELIGH, G. S. (1952).—Factors influencing the performance of the precipitin test in the determination of blood meals of insects. *Canad. J. Zool.* **30**: 213-18.

FISK, F. S., and SHAMBAUGH, G. F. (1952).—Protease activity in adult *Aedes aegypti* mosquitoes as related to feeding. *Ohio J. Sci.* **52**: 80-8.

HOSKING, K. S., and MACINNES, D. G. (1948).—Notes on the bionomics of *Anopheles gambiae* and *Anopheles funestus* in East Africa. *Bull. Ent. Res.* **39**: 453-65.

HOVANITZ, W. (1947).—Physiological factors which influence the infection of *Aedes aegypti* with *Plasmodium gallinaceum*. *Amer. J. Hyg.* **45**: 67-81.

HUFF, C. G. (1934).—Comparative studies on size, variability, and growth rate of oocysts of different strains of avian malaria. *Amer. J. Hyg.* **19**: 123-47.

LEE, D. J., CLINTON, K. J., and O'GOWER, A. K. (1954).—The blood sources of some Australian mosquitoes. *Aust. J. Biol. Sci.* **7**: 282-302.

PROOM, H. (1943).—The preparation of precipitating sera for the identification of animal species. *J. Path. Bact.* **55**: 419-26.

SHLENOVA, M. F. (1938).—Vitesse de la digestion du sang par la femelle de *l'Anopheles maculipennis messeae* aux temperatures effectives constantes. (In Russian.) *Med. Parasitol., Moscow* **7**: 716-35. (Abstr.: *Rev. Appl. Ent.* B **27**: 174 (1939)).

WEITZ, B., and BUXTON, P. A. (1953).—The rate of digestion of blood meals of various haematophagous arthropods as determined by the precipitin test. *Bull. Ent. Res.* **44**: 445-50.

WEST, A. S., and ELIGH, G. S. (1952).—The rate of digestion of blood in mosquitoes. Precipitin test studies. *Canad. J. Zool.* **30**: 267-72.

WIGGLESWORTH, V. B. (1943).—The fate of haemoglobin in *Rhodnius prolixus* (Hemiptera) and other blood-sucking arthropods. *Proc. Roy. Soc.* B **131**: 313-39.

THE INFLUENCE OF ENVIRONMENTAL TEMPERATURE ON THE LEVEL OF PLASMA ANTIDIURETIC SUBSTANCES IN THE RAT

By Kathleen W. Robinson* and W. V. Macfarlane*

[*Manuscript received August 5, 1955*]

Summary

The level of plasma antidiuretic substances (ADS) was found to be doubled when rats were exposed continuously to an atmosphere of 95°F (35°C) and 30 mm Hg water vapour pressure for 28 days, and it was reduced to half with continuous exposure to cold (43°F (6·5°C)). When reduction in body weight accompanied heating, the antidiuretic potency of the blood was markedly increased.

Exposure to a hot atmosphere (104°F (40°C), 30 mm Hg water vapour pressure) for 2 hr, whether acutely or once daily for 28 days, failed to alter the concentration of ADS in the blood. A single exposure to these conditions for 4 hr produced a four-fold rise in ADS level.

The release of ADS appears to arise from dehydration induced by heat and not from the response of receptors to heat as such. Plasma protein concentration changed in the same direction as ADS concentration.

Acclimatization to heat or cold did not alter the sensitivity of the rat's kidney tubules to exogenous pitressin.

Adrenalectomy failed to produce any change in antidiuretic activity of the blood during short, acute, heat exposure.

I. Introduction

A homeotherm, relying largely on evaporative cooling to adjust body temperature in a hot environment, has adaptive mechanisms to prevent dehydration of the cells. These mechanisms are not fully understood. Water is retained by reduction of urine volume in man and animals during heat exposure (Weiner 1944; Hellmann and Weiner 1953). Peripheral vasodilatation, which accompanies heating, produces a compensatory fall in blood flow to the kidney (Radigan and Robinson 1949; Kenney 1952), but this blood shift is not alone responsible for reduced urine production. The release of antidiuretic hormone (ADH) from the neurohypophysis into the blood stream is a further possible means of conserving body water in a hot environment.

Several observers have noted a rise in antidiuretic substances (ADS) in human urine after heavy sweating (Hellman and Weiner 1953; Itoh and Kimura 1954). Recently, Itoh (1954) reported that the antidiuretic potency of rat serum, following a short exposure, showed a rise with heat and a fall with cold.

In an attempt to obtain further information on the effect of heat on blood levels of ADS, the ADS activity of rat's plasma was measured following (i) continued exposure over a period of 1 month to hot and to cold environments, and (ii) short term exposure to heat. The exposure time in (i) was considered sufficient to acclimatize the animals to the temperature conditions, since Sellers, Reichman, and Thomas (1951) have shown that acclimatization to cold is fully developed in rats after 4 or 5 weeks and there is evidence that acclimatization to heat is also rapid (Beattie and Chambers 1953). Changes in the amount of ADS produced in acclimatization could thus be studied.

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Birnie *et al.* (1949) and Birnie (1953) have claimed that the blood of adrenalectomized animals has an antidiuretic potency higher than that of intact rats because the liver destroys less ADH, while reduced renal clearance following adrenalectomy (Ginsburg 1954), or unknown causes (Mirsky, Stein, and Paulisch 1954) are considered by some workers to raise the ADS level. Others disagree with this concept (Ames and van Dyke 1952; Leaf *et al.* 1952). It was considered that, if adrenalectomy hinders the removal of ADS from the blood, the effect would be magnified in heated animals in which the production of these substances is increased. Therefore, a further experiment was designed to compare the levels of plasma ADS in adrenalectomized rats with normals during acute heat exposure.

II. Methods

Four series of experiments were conducted:

(*a*) *Continuous Exposure*

Male albino rats of similar weight (200 g) and age, from an inbred Wistar strain, were divided into three groups: one group was exposed continuously for 28 days in a hot-box to a temperature of 35°C; a second group was kept continuously over this period in a lighted refrigerator at 6·5°C; and a third group was exposed to external temperature conditions (av. monthly 3 p.m. temperature 22°C). The blood of all these animals was assayed for antidiuretic activity and for plasma protein concentration. This experiment took place in September-October. It was repeated (with omission of the cold exposure group) on other rats in March-April (av. monthly 3 p.m. temperature 27·2°C).

(*b*) *Repeated Heat Exposure*

A group of rats of the same sex, age, and weight as used in Section II (*a*) was exposed daily for 2 hr to an atmosphere of 40°C and 30 mm Hg vapour pressure for 28 days. The plasma ADS and protein levels were compared with a group of control rats maintained on the same level of nutrition at normal environmental temperatures.

(*c*) *Acute Heat Exposure*

Male rats were subjected to one acute exposure at 40°C and 30 mm Hg vapour pressure. The plasma antidiuretic activity and the plasma protein concentration was measured after 2 and 4 hr in the heat.

(*d*) *Adrenalectomy*

The plasma ADS of a group of pair-fed controls and of a group of rats, adrenalectomized 10 days previously and fed no salt supplement, were compared after 2 hr exposure to 40°C and 30 mm Hg vapour pressure.

In all these experiments drinking water was allowed until 3 hr before blood assays were made.

Antidiuretic activity of plasma was determined by a modification of the method of Dicker (1953) in which intravenous injections are made into an assay rat under ethanol anaesthesia with a constant water load. The method is sensitive to injections of 10 μU of pitressin. Male rats weighing *c.* 200 g were starved for 16 hr prior to the test, and then hydrated with an oral dose of alcohol-water (12 per cent. ethanol solution) equivalent to 5 per cent. of body weight. Thirty minutes later a similar dose of water at body temperature was administered. The bladder was catheterized, the external jugular vein cannulated with 1 mm diameter polythene tube, and an indwelling stomach tube inserted. Urine samples were collected in a 5-ml measuring cylinder graduated to 0·05 ml and measurements were made at 10-min intervals. The animal was kept constantly hydrated with 2 per cent. ethanol solution by stomach tube, the amount administered from a microburette at 10-min intervals being equal in volume to the urine voided. When a steady rate of urine flow had been obtained (0·15-0·2 ml/min), intravenous injections of plasma from the experimental animal were made into the jugular cannula (volume = 0·4 ml plasma washed through with 0·1 ml of 0·85 per cent. saline). The antidiuretic response was measured by comparing the volume of urine excreted in the 20 min following injection with that for the same period just prior to injection. The antidiuretic effect was then compared with that obtained from known amounts of pitressin (Parke, Davis and Co., No. 161) injected into the same assay rat.

TABLE 1

PLASMA ADS AND PROTEIN CONCENTRATION OF RATS EXPOSED CONTINUOUSLY FOR 28 DAYS TO HOT AND COLD ATMOSPHERES

	Cold Exposure 43°F (6·5°C)	Heat Exposure 95°F (35°C)	Control	Season
No. of animals	8	6*	8	Sept.–Oct.
Plasma ADS (μU/ml)	42±35	182±24	80±41	
t†	$P = 0{\cdot}05$	$P < 0{\cdot}001$		
No. of animals	—	10	10	March
Plasma ADS (μU/ml)	—	52±15	25±12	
t†	—	$P < 0{\cdot}001$		

* Loss of body weight over the period averaged 16 per cent. of the initial value. Body weight remained constant in all other groups.

† Probability of difference of ADS level from controls.

Blood was collected in a heparinized tube from the neck vessels of experimental rats by rapid decapitation. It was quickly centrifuged, and the plasma was injected into the assay animal within 15 min of being withdrawn. The plasma protein concentration of the blood was measured by an Abbé refractometer.

III. RESULTS

(*a*) *Continuous Exposure to Heat or Cold*

Plasma from rats kept continuously in a hot atmosphere for 28 days during the months September-October had an antidiuretic potency more than twice that of control rats, while rats kept in the cold for this same period had about half the value found in controls. This is illustrated in Table 1 and Figure 1. Statistical examination of the results by the *t*-test, showed that values for heat-exposed rats were highly significantly different from the controls ($P < 0{\cdot}001$), while those exposed to cold were probably significantly different ($P = 0{\cdot}05$).

As judged by plasma protein values, blood of the rats exposed to heat showed 4·9 per cent. concentration relative to the controls, while in those exposed to the cold there was 6·6 per cent. dilution of plasma proteins. The protein concentration was recorded as an indirect measure of plasma water.

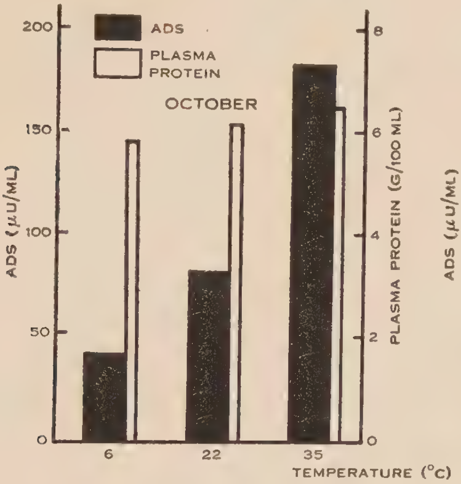

Fig. 1.

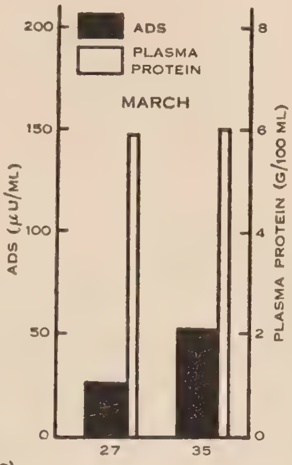

Fig. 2.

Fig. 1.—Histogram relating the mean ADS levels in the plasma of rats kept during summer at 6°, 22°, and 35°C. to the mean plasma protein concentrations simultaneously determined. Heat increased ADS and plasma concentration, cold decreased both quantities.

Fig. 2.—ADS and plasma protein in rats kept at 27° and 35°C during autumn. The ADS levels are lower than those recorded in Figure 1, but they vary similarly with plasma protein concentration.

Additional evidence of the high antidiuretic potency of the plasma of the heat-acclimatized rats was provided when attempts were made to produce a water diuresis in some of their number. The hydration was carried out under prevailing atmospheric conditions, and commenced immediately after the animals were removed from the hot environment. Oral administration of water in amounts which were diuretic in both cold-acclimatized and control rats failed to increase urine flow above 0·3 ml/hr in the heat-adapted animals (Table 2).

In this experiment, while both control and cold rats maintained body weight over the experimental period, that of rats exposed to heat fell an average of 16 per cent. during the 28 days.

In contrast, when the experiment was repeated in March-April, body weight was maintained at the initial value in both heat-exposed and control rats (Fig 2). Results then showed that although the antidiuretic potency of the blood of hot rats was significantly above the controls ($P < 0{\cdot}001$), the rise was much less than in the earlier experiment. Plasma protein levels of both groups were comparable (6·0±0·3 and 5·9±0·4 respectively).

TABLE 2

RESPONSE OF HOT- AND COLD-ACCLIMATIZED RATS TO A WATER LOAD

Atmosphere	Water Load (ml)	Urinary Output (ml/hr)	Period of Time (hr)	Fluid Retained by Body (ml)
Hot*	29	0·31	7	27
	23	0·30	5·5	21
Cold†	22	3·3	6	2
	25	3·5	6	4
Control	36	3·8	7	9
	25	3·0	6	7

* 28 Days at 35°C. Body weight reduced by 16 per cent. over the period.
† 28 Days at 6·5°C.

The difference in ADS values for controls in the two experiments may possibly be explained as a seasonal fluctuation. This is suggested by data (unpublished) from other experiments at present in progress.

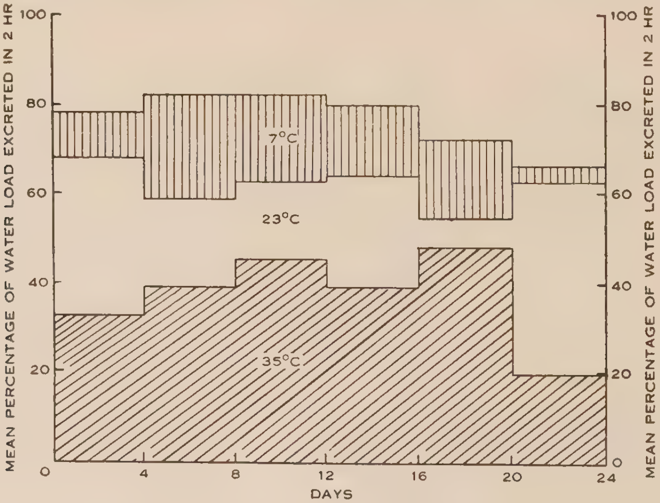

Fig. 3.—Water excretion during 2 hr after a water load of 5 per cent. of body weight, expressed as a percentage of the water administered, in a series of 180-g male Wistar rats. Cold-acclimatizing rats excreted a mean of 75 per cent., and heat-acclimatizing rats only 40 per cent. of the water load in 2 hr. Groups comprised 15 rats.

In Figure 3 is shown the mean percentage of water load excreted in 2 hr by 180-g male Wistar rats when tested periodically under three different atmospheric conditions to which the animals were exposed continually for 24 days. Values are means for 15 rats in each group. Percentage of water load excreted bears an inverse relationship to the values found for ADS levels in these heat-adapted, cold-adapted, and control animals.

TABLE 3

SENSITIVITY TO PITRESSIN OF CONTROL, HOT-, AND COLD-ACCLIMATIZED RATS

Pitressin Injection (μU)	Per Cent. Reduction in Urine Volume (mean of 2 animals in each group)		
	Control	Heat-acclimatized	Cold-acclimatized
20	30	28	26
40	50	46	47
60	62	60	61
80	73	72	70

Table 3 illustrates the reliability of the assay method and its independence of the prior treatment of the rats. The sensitivity to injections of graded doses of pitressin of cold-acclimatized, heat-acclimatized, and control rats used as assay animals on a constant 10 ml/100 g body weight load, is essentially the same in all groups. The greatest difference is 13 per cent. and the average is 5 per cent.

(b) *Repeated and Acute Heat Exposure*

Daily exposure of rats for a short period (2 hr) to a hot atmosphere (40°C, 30 mm Hg vapour pressure), although more severe heating than that used in the continuous exposure experiment, failed to produce a rise in level of blood ADS in 28 days. A single day's exposure, however, to this same hot temperature for a longer period (4 hr) was effective in increasing the ADS of the blood sample (Table 4 and Fig. 4). A *t*-test analysis indicated that this difference between the 2-hr and the 4-hr exposures was highly significant ($P<0{\cdot}001$).

TABLE 4

ANTIDIURETIC POTENCY AND PROTEIN CONTENT OF RAT PLASMA FOLLOWING SINGLE AND REPEATED EXPOSURE TO A HOT ATMOSPHERE (104°F (40°C), 30 MM HG VAPOUR PRESSURE)

Condition of Animals	Time of Exposure to Heat	No. of Animals	ADS (μU/ml)	Plasma Protein (g/100 ml)	Rectal Temperature (°C)
Normal	2 Hr daily for 28 days	7	28±15	5·9	—
,,	2 Hr single exposure	9	30±16	6·0	39·5
,,	4 Hr single exposure	5	133±29	6·9	40·9
,,	Control	6	27±18	5·9	36·8
Adrenalectomized	2 Hr single exposure	5	29±12	6·0	—

Plasma protein levels were raised (to 6·9 g/100 ml) after 4 hr at 40°C but remained near the control level of 5·9 g/100 ml with only 2 hr of heating.

(c) *Adrenalectomy*

Adrenalectomy did not increase the concentration of ADS nor alter plasma protein levels during a 2 hr exposure to heat (Table 4 and Fig. 4).

IV. Discussion

Itoh (1954) suggested that the release of ADH from the posterior pituitary gland of rats, which is accelerated by exposure to heat and inhibited on exposure to cold, results not from dehydration induced by heat but rather from heat *per se* acting by some mechamism other than dehydration.

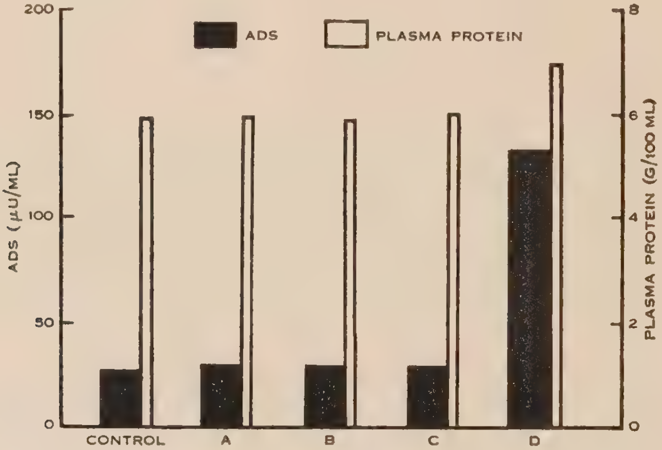

Fig. 4.—Plasma protein and ADS were unaffected by 2 hr at 40°C (*A*), by adrenalectomy and heating for 2 hr (*C*), and by daily exposure for 2 hr to 40°C for 28 days (*B*). Four hr at 40°C (*D*) tripled the ADS concentration and raised the plasma protein level from 6·0 to 6·9 g/100 ml.

The results of the present investigation do not, however, support such a concept. Although a 2-hr exposure of rats to 40°C raised body temperature from a mean value of 36·8 to 39·5°C, no increase in blood ADS was observed. It seems reasonable to expect that a 2·7°C rise in body temperature should provide sufficient stimulus for the activation of any heat-sensitive mechanism for ADH release. Only when some concentration was apparent (as indicated by plasma protein levels) following a longer exposure of 4 hr to this atmosphere did plasma ADS increase. Under these atmospheric conditions a 200-g rat loses water by evaporation at the rate of 5 g/hr (unpublished data). The loss of 10 g after 2 hr heating is evidently insufficient to produce a noticeable threat to the water reserves, but when it amounts to 20 g (approx. 1/6 of the body water), an adaptive water conservation release of ADS is initiated. Furthermore, when rats were subjected to continuous long-term exposure to heat and cold, the amount of ADS in the blood closely paralleled the plasma protein level. On the other hand, repeated daily 2-hr exposures to a high temperature did not cause dehydration. Apparently compensation by water replacement during the periods at room temperature took place, since no evidence of haemoconcentration was observed. The parallel changes in plasma ADS and plasma protein in different thermal environments are illustrated in Figures 1, 2, and 4.

Evaporative water loss initially involves reduction of plasma volume, and this concentrates the plasma electrolytes as well as plasma proteins. Subsequent extrusion of water from the cells may reduce plasma dehydration. The situation is made more complex by migration of sodium into, and potassium out of, cells during long heating (unpublished data). There is, however, concentration of plasma proteins when the plasma electrolyte osmotic pressure is raised. Plasma proteins seem to be an index of plasma water, though there is no evidence that the protein osmotic pressure is the effective stimulus to ADH release.

It is interesting to compare the high values for ADS in the September-October experiment, involving continuous exposure to heat, when 16 per cent. reduction of body weight occurred, with the smaller rise that followed heating when the experiment was repeated at the same temperature without loss of weight. Other workers have reported higher than normal ADS blood levels in animals and men fed protein deficient rations (Leslie and Ralli 1947; Schweppe and Freeman 1951; Guggenheim and Hegstead 1953), and *in vitro* studies would suggest (Birnie, Blackmore, and Heller 1952) that the protein-deficient liver destroys ADH less rapidly than the normal liver. Guggenheim has demonstrated that rats maintained on a thiamine-deficient diet exhibit a delayed diuretic response to a water load with impairment of ability of the liver to inactivate pitressin (Guggenheim 1954*a*), although the derangement of water metabolism in pyridoxine- and pantothenic acid-deficient rats appears to be related to interference with adrenocorticotrophic hormone production rather than to ADH accumulation in the body (Guggenheim 1954*b*).

To explain the difference in our results between the two continuous heat-exposure experiments it is suggested that, in the earlier series, two factors were operating to raise the ADS level of the plasma: (i) heat acting through the stimulus of haemoconcentration to increase the output of ADS, and (ii) malnutrition resulting in an impairment of normal ADS destruction. In the latter series, factor (ii) was removed and the antidiuretic potency of the blood fell correspondingly.

The sensitivity of the kidney to pitressin, and it is presumed, to ADH, in heat- and cold-acclimatized rats appears to be the same as that of rats living in thermally neutral environments. Adaptation of an animal to heat does not involve change in kidney response to ADH, but rather in the synthesis or release of the hormone as regulated by environmental factors. Hydration tests would indicate that differences in ADS levels of heat-adapted, cold-adapted, and control rats are related to differences in their respective rates of excretion of a water load.

No delay in removal of ADS from the blood of adrenalectomized rats was apparent under the conditions of these experiments. Animals, from which both adrenals had been removed 10 days previously, exhibited the same ADS response to 2 hr acute heat exposure as the controls. This is in agreement with the findings of Ames and van Dyke (1952) and it seems to be the result obtained when intravenous rather than intraperitoneal injection is used in the assay procedure.

The findings of the present investigation, indicate that ADS are released into the blood of rats following heat exposure. This occurs only after the amount of water lost in evaporative cooling is sufficient to produce a significant, although small, change in water reserves. Plasma protein concentration gives an indirect measure of this change. The ADS appears in both long-term and acute heat exposure, in amounts depending on the immediate extracellular water concentration. There is a possibility that the intracellular water reserve may be involved in seasonal ADS release. It would be expected that in sweating mammals, which rely on evaporative cooling to maintain body temperature in hot climates, the release of ADS would play an even more important role in heat adaptation than is seen in the rat. Experiments on man in this context are being carried out.

V. Acknowledgment

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VI. References

Ames, R. A., and van Dyke, H. B. (1952).—*Endocrinology* **50**: 350.

Beattie, J., and Chambers, R. D. (1953).—*Quart. J. Exp. Physiol.* **38**: 55.

Birnie, J. H. (1953).—*Endocrinology* **52**: 33.

Birnie, J. H., Blackmore, K. E., and Heller, H. (1952).—*Experientia* **8**: 30.

Birnie, J. H., Jenkins, R., Eversole, W. J., and Gaunt, R. (1949).—*Proc. Soc. Exp. Biol., N. Y.* **70**: 83.

Dicker, S. E. (1953).—*J. Physiol.* **122**: 149.

Ginsburg, M. (1954).—*J. Endocrinol.* **11**: 165.

Guggenheim, K. (1954*a*).—*Metabolism* **3**: 44.

Guggenheim, K. (1954*b*).—*Endocrinology* **55**: 156.

Guggenheim, K., and Hegsted, D. M. (1953).—*Amer. J. Physiol.* **172**: 23.

Hellmann, K., and Weiner, J. S. (1953).—*J. Appl. Physiol.* **6**: 194.

Itoh, S. (1954).—*Jap. J. Physiol.* **4**: 185.

Itoh, S., and Kimura, M. (1954).—*Jap. J. Physiol.* **3**: 277.

Kenney, R. A. (1952).—*J. Physiol.* **118**: 25P.

Leaf, A., Mamby, A. R., Rasmussen, H., and Marasco, J. P. (1952).—*J. Clin. Invest.* **31**: 914.

Leslie, S. H., and Ralli, E. P. (1947).—*Endocrinology* **41**: 1.

Mirsky, A., Stein, M., and Paulisch, G. (1954).—*Endocrinology* **54**: 491.

Radigan, L. R., and Robinson, S. (1949).—*J. Appl. Physiol.* **2**: 185.

Schweppe, J. S., and Freeman, S. (1951).—*Proc. Soc. Exp. Biol., N.Y.* **77**: 51.

Sellers, E. A., Reichman, S., and Thomas, N. (1951).—*Amer. J. Physiol.* **167**: 644.

Weiner, J. S. (1944).—*J. Physiol.* **103**: 36P.

MICROMETHOD FOR THE ESTIMATION OF GLYCOGEN IN THE GENITAL ORGANS OF THE MOUSE

By Judith H. Balmain,* J. D. Biggers,* and P. J. Claringbold*

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Summary

(i) A micromethod has been described for the estimation of small amounts of glycogen in the vagina and uterus of the mouse.

(ii) The method involves digesting the tissue in KOH, precipitating with 60 per cent. ethanol, and extracting glycogen from the precipitate with 5 per cent. trichloroacetic acid. The glycogen is estimated with the anthrone reagent.

(iii) The method was satisfactory for estimating amounts of glycogen down to 5 μg in the presence of tissue hydrolysates made from the mouse vagina. A quantitative recovery of the glycogen was obtained.

I. Introduction

Various methods have been described for the determination of glycogen in small amounts of tissue and in tissue of low glycogen content, e.g. Good, Kramer, and Somogyi (1933); Zondek and Shapiro (1942); Boettiger (1946); Seifter *et al.* (1950); Walaas and Walaas (1950); van der Kleij (1951); Walaas (1952*a*, 1952*b*); Bowman (1953); Fong, Schaffer, and Kirk (1953); Kemp and van Heijningen (1954), and van der Vies (1954). The authors of all of these papers, except Fong *et al.* (1953) were concerned with estimating glycogen in amounts greater than 100 μg. The aim of the present work was to produce a method capable of estimating the very small amounts of glycogen which are present in the vagina and the uterus of the mouse. Experience has shown that, depending on the degree of stimulation by oestrogens, these quantities are aften as low as 5 μg and are rarely greater than 100 μg (Balmain, Biggers, and Claringbold 1956).

In most of the methods, the tissue is digested in strong potassium hydroxide solution and the glycogen is precipitated by the addition of ethanol. Recent workers have favoured the anthrone-reducing sugar method for the estimation of the precipitated glycogen, since the anthrone reagent reacts directly with polysaccharides, e.g. glycogen, and its use eliminates a hydrolytic stage. The method we have evolved follows these principles, and is modelled on the micromethod of Seifter *et al.* (1950). Various aspects of the method, including an examination of the "blank" due to interfering substances, have been studied using multifactor experimentation (Fisher 1949; Bennett and Franklin 1954).

II. Experimental

(*a*) *Preparation of Tissues*

Randomly-bred albino mice were used in this work. They were killed by dislocation of the spine, and the genital tract was then dissected out as rapidly as possible. After removal of extraneous fat, the vagina and the uterine horns were separated from the cervix, and the urethra was stripped from the vaginal wall.

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(b) *Estimation of the Glycogen by the Anthrone Reaction*

Glycogen was estimated colorimetrically with the anthrone reagent, following closely the method of Seifter *et al.* (1950). However, the volumes of reagents used were reduced in order to allow smaller quantities of glycogen to be estimated.

The anthrone reagent (0·2 per cent. solution in 95 per cent. sulphuric acid) is freshly prepared each day.

The glycogen is dissolved in 2·5 ml of distilled water or 5 per cent. trichloroacetic acid (TCA) and 5·0 ml of the anthrone reagent added. The tubes containing the glycogen solution are agitated in cold running water throughout the addition of the anthrone reagent; the reacted solutions are then heated for 10 min in a boiling water-bath, cooled, and the percentage transmission read at 620 mμ in a Coleman spectrophotometer.

Transmission readings between 15 and 60 per cent., which cover the accurate range of the spectrophotometer, are given by 20-100 μg glycogen per tube. The columns headed "unprecipitated" in Table 1 contain examples of calibration curves. Log transmission is linearly related to the concentration of glycogen. However, secular shifts in the position of the calibration curve are a characteristic finding, and thus it is essential to determine a calibration curve for each experiment.

In tissue samples where the glycogen content is less than 20 μg it is necessary to boost the solutions of extracted glycogen by the addition of a known quantity of glycogen. When low levels of glycogen are expected, either 20, 30, or 40 μg of glycogen in 0·4 ml of water are added to the dissolved extracted glycogen, and at the same time the total volume of the glycogen solution is adjusted to 2·5 ml.

(c) *Recovery of Glycogen from Aqueous Solution* (*Table* 1)

An experiment was designed to study the precipitation with 60 per cent. ethanol of amounts of glycogen of less than 100 μg. Preliminary experiments had shown that the volume (up to 0·5 ml) of KOH in which the glycogen is dissolved is not critical for the recovery of glycogen. It had also been found that the speed of centrifugation is important for the collection of the precipitated glycogen, appreciable losses occuring with speeds less than 2500 r.p.m. The details of the experiment are shown in Table 1.

The sets of four tubes corresponding to each glycogen level on each day are prepared from a common glycogen solution, and the results are therefore correlated. The experiment is thus of the split-plot type and calls for a special form of analysis (Cochran and Cox 1950). The analysis of variance is divided into two parts, each having a separate error term. The day and glycogen effects are examined from the plot totals and an inter-plot error, while the treatment effects are tested against an intra-plot error. The analysis shows that a large difference exists between the mean results on each day. The calibration curve is parallel on the 2 days and is linear over the range of glycogen levels studied. No difference is observed between the unprecipitated and the precipitated material, and thus the addition of 60 per cent. ethanol leads to quantitative precipitation of glycogen from aqueous solution. This differs from the results of Walaas (1950*a*, 1950*b*) who found that losses of glycogen occurred in the precipitation process.

The experiment has not been repeated for the glycogen range of 5-20 μg since a final check of the method includes this range (see below). The highly significant day difference indicates that it is essential to determine a separate calibration curve on each day.

TABLE 1

RECOVERY OF GLYCOGEN FROM AQUEOUS SOLUTIONS

Glycogen (μg)	Optical Density							
	Day 1				Day 2			
	Unprecipitated		Precipitated		Unprecipitated		Precipitated	
20	1·79	1·79	1·81	1·83	1·83	1·84	1·83	1·84
30	1·70	1·67	1·71	1·68	1·73	1·68	1·76	1·75
40	1·58	1·56	1·62	1·62	1·65	1·68	1·60	1·65
50	1·50	1·46	1·50	1·47	1·54	1·58	1·54	1·60
60	1·40	1·41	1·45	1·36	1·46	1·49	1·44	1·48
70	1·34	1·32	1·32	1·30	1·37	1·37	1·38	1·39
80	1·23	1·32	1·25	1·20	1·26	1·32	1·29	1·28
90	1·11	1·28	1·15	1·15	1·20	1·11	1·20	1·24
100	1·04	1·00	1·04	1·04	1·08	1·13	1·15	1·16

Analysis of Variance

Source of Variation	D.F.	Mean Square†	*F*
Between days	1	50·1	35·8***
Between glycogen levels:	(8)		
Linear	1	392·8	2800***
Remainder	7	0·6	< 1
Inter-plot error	8	1·4	
Between treatments	1	0·9	< 1
Days × treatments	1	1·0	< 1
Glycogen levels × treatments	8	0·7	< 1
Intra-plot error	44	1·25	

*** $P <$ 0·001. † Mean squares have been multiplied by 1000.

(d) *Substances which Interfere with the Estimation of Tissue Glycogen*

Seifter *et al.* (1950) pointed out that proteins which are precipitated along with glycogen in 60 per cent. ethanol are likely to cause an over-estimation of glycogen when reducing sugar methods are used. The amount of interference is smaller with the anthrone reagent than with other methods of sugar estimation, as tryptophan is the only amino acid giving a colour with anthrone at 620 mμ (Seifter *et al.* 1950; Sheltar 1952). It is also possible that other substances such as hexose sugars and related compounds may interfere, as these give a colour reaction with the anthrone reagent (Sattler and Zerban 1950; Sheltar 1952; Hill, Hawkins, and Strasser 1953). However, since Little (1949) and Young and Raisz (1952) have shown that free reducing sugar is completely destroyed by heating with alkali, protein material seems likely to be the most serious contaminant in the present method.

Table 2

THE REDUCTION OF THE BLANK BY TCA EXTRACTION

Test No.	Glycogen Equivalent of Blank (μg)			
	No TCA Extraction		TCA Extraction	
1	49	44	38·5	35
2	18	21	12	16
3	9·5	7	6	5·5
4	8	14·5	4	5·5
5	12	12	9·5	8·5
6	17	13	12·5	12
7	12·5	18	12	13·5
8	9	5	7	9·5
9	9	13	9	6·5
10	17	7	7	6
11	8	6·5	6	7
12	7	11	9	8
13	10	11·5	9	8

Analysis of Variance

Source of Variation	D.F.	Mean Square	F
Between tissue samples	12	186·6	19·2***
Between treatments	1	171·9	17·7***
Samples × treatments	12	11·4	1·17
Error	26	9·72	

*** $P <$ 0·001.

Several workers have attempted to purify glycogen by dissolving it again and reprecipitating it with 60 per cent. ethanol (Seifter *et al.* 1950; Walaas 1952*a*, 1952*b*; Bowman 1953). In our experience this procedure was found to reduce the "blank" by only a small amount. Increasing the concentration of KOH beyond 30 per cent. lowered the blank but also affected the recovery of glycogen. However, increasing the volume of KOH solution from 0·2 ml as used by Walaas (1952*a*, 1952*b*), to 0·4 ml significantly reduced the blank while it did not affect the recovery of glycogen. A volume of 0·4 ml has thus been used throughout this work.

(e) *Reduction of Tissue Blank by Trichloroacetic Acid Extraction* (*Table* 2)

If tissues are incubated at 37°C in phosphate-Ringer solution (pH 7·4), the glycogen breaks down due to enzyme action. When the tissue is then extracted for glycogen, and the anthrone reaction is carried out, a considerable blank is obtained, presumably due to non-specific protein substances (see below).

Since TCA dissolves glycogen and precipitates proteins, it was introduced into the method to see if it reduced the blank. The experiment described in Table 2 demonstrates the effect of TCA extraction of the ethanol precipitate. The analysis of variance shows that the difference between the tubes treated and those not treated with TCA is highly significant. The use of TCA leads to a 23·5 per cent. reduction of the blank, and since the samples × treatments mean square is not significant, the reduction of the blank may be considered uniform for all samples.

(*f*) *Preparation of Tissue for the Study of the Blank* (*Fig.* 1)

The TCA extraction technique was used to follow the breakdown of glycogen in the vagina of the mouse during incubation at 37°C in phosphate-Ringer (pH 7·4).

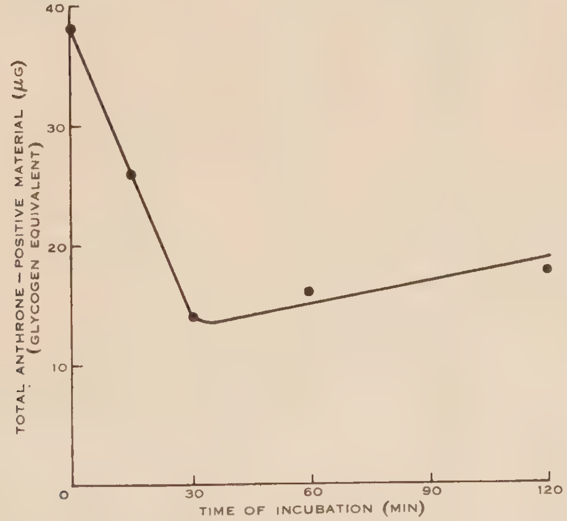

Fig. 1.—The breakdown of glycogen in mouse vaginae during incubation in phosphate-Ringer at 37°C.

The design and results of the experiment are shown in Figure 1. There is a rapid reduction in the quantity of anthrone-positive material during the first 30 min, after which no further reduction takes place. The initial fall is due to glycogen breakdown, and the residual material may be attributed to interfering substances. The slight rise occurring after 30 min is statistically significant ($0 \cdot 01 > P > 0 \cdot 001$) but its cause is unknown. Its presence stresses the necessity of incubating the tissue for a constant time in the determination of non-glycogen substances.

In a further experiment, the blanks of vaginae incubated at 37°C in phosphate-Ringer and in phosphate-Ringer containing 0·2 ml saliva were compared. No significant difference was found between them ($t_{(8)} = 1 \cdot 08$, $P > 0 \cdot 05$), which confirms the non-glycogen nature of the blank.

(*g*) *Quantitative Recovery of Glycogen in the Presence of Tissue* (*Table* 3)

The experiment shown in Table 3 was designed to examine the quantitative recovery of glycogen in the presence of tissue with the TCA extraction procedure. The linear regression of log transmission on concentration of glycogen is highly significant when compared with the deviations from linear regression (remainder).

TABLE 3

RECOVERY OF GLYCOGEN IN THE PRESENCE OF HYDROLYSED GLYCOGEN-FREE TISSUE

Glycogen (μg)	Optical Density			
	Unprecipitated Glycogen plus Precipitated Tissue		Precipitated Glycogen plus Precipitated Tissue	
5	1·71	1·69	1·71	1·69
10	1·66	1·66	1·59	1·63
15	1·53	1·62	1·56	1·57
20	1·58	1·57	1·59	1·56
25	1·48	1·52	1·52	1·52
30	1·45	1·44	1·40	1·47
35	1·44	1·45	1·33	1·40
40	1·39	1·39	1·27	1·34
45	1·30	1·32	1·32	1·31
50	1·20	1·21	1·28	1·26

Analaysis of Variance

Source of Variation	D.F.	Mean Square	*F*
Between glycogen levels	(9)		
Linear	1	805·0	1103***
Remainder	8	1·0	1·4
Between treatments	1	2·4	3·3
Glycogen levels × treatments	(9)		
Linear	1	0·2	<1
Remainder	8	2·3	3·2*
Error	20	0·73	

* $0·05 > P > 0·01$; *** $P < 0·001$.

However the glycogen × treatment mean square is also just significant. Further analysis shows this to be due to high-order, opposed, curvature effects of unknown origin. The presence of this effect, however, does not invalidate the conclusion that there is no significant difference between the treatments at the $P = 0·05$ level. Thus there is adequate recovery of glycogen in the presence of hydrolysed tissue, and the method may be considered satisfactory for the microestimation of glycogen.

III. PROPOSED METHOD FOR THE MICROESTIMATION OF GLYCOGEN

(*a*) *Reagents*

Potassium hydroxide: 30 per cent. aqueous solution.

Absolute ethanol.

Trichloroacetic acid: 5 per cent. aqueous solution.

Sulphuric acid: 95 per cent. aqueous solution, prepared by adding 1 l. A.R. sulphuric acid (S.G. 1·84) to 50 ml distilled water.

Anthrone reagent: 0·2 per cent. solution of anthrone (B.D.H.) in 95 per cent. sulphuric acid, prepared each day.

(*b*) *Procedure*

The vagina is bisected so that one-half may be used for total glycogen, and the other half for a blank or other determination. Similarly each uterine horn can be used for separate determinations. The piece of tissue is mopped roughly dry on blotting-paper, weighed quickly on a torsion balance (capacity 200 mg), and placed in a small tube (capacity 2 ml) containing 0·4 ml 30 per cent. KOH. The tube is then heated at 100°C for 30 min. The time taken from the death of the animal to placing the tissue in KOH is less than 2 min.

After digestion of the tissue with KOH, the glycogen is precipitated by the addition of 0·8 ml absolute ethanol giving a final concentration of about 60 per cent. Thorough mixture of the solutions is ensured by means of a thin glass rod, which is reserved for later steps. The ethanol mixture is heated to boiling in a water-bath and cooled for 5-10 min in a refrigerator. Glycogen is removed from this suspension by centrifugation at 3500 r.p.m. for 30 min. The supernatant is then decanted off and the tubes drained for at least 15 min on blotting-paper. When large numbers of estimations are being made it is convenient to store the precipitated glycogen at this stage by keeping the tubes in a refrigerator overnight.

Glycogen is obtained quantitatively from the microtubes by two successive extractions with 5 per cent. TCA, using 1·0 ml for the first washing and 1·5 ml for the second (total 2·5 ml). For each washing the TCA is added to the tubes, the mixture being thoroughly agitated by means of the stirring-rod used previously. The tubes are then centrifuged at 3500 r.p.m. for 20 min, and the supernatants are decanted carefully into 6 × 1 in. boiling tubes, each microtube being well drained.

The solutions collected in the boiling tubes are then subjected to the anthrone reaction as described earlier. The values obtained give an estimate of the "total glycogen", which will consist of "true glycogen" together with ethanol-precipitated interfering substances (i.e. the blank).

If the true glycogen content of the tissue is required, then a similar procedure is carried out on the other half of the tissue after incubation at 37°C in phosphate-Ringer (pH 7·4) for 60 min, in order to obtain an estimate of the blank. The difference between the total glycogen and the blank gives an estimate of the true glycogen content of the tissue. By the adoption of suitable randomization procedures an estimate of the true glycogen content of an organ may be made without taking exactly equal weights of tissue for the analysis of total and blank glycogen (see Balmain *et al.* 1956).

IV. ACKNOWLEDGMENTS

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V. REFERENCES

BALMAIN, J. H., BIGGERS, J. D., and CLARINGBOLD, P. J. (1956).—*Aust. J. Biol. Sci.* **9**: 147.

BENNETT, C. A., and FRANKLIN, N. L. (1954).—"Statistical Analysis in Chemistry and the Chemical Industry." (John Wiley & Sons Inc.: New York.)

BOETTIGER, E. G. (1946).—*J. Cell. Comp. Physiol.* **27**: 1.

BOWMAN, R. H. (1953).—*J. Physiol.* **172**: 157.

COCHRAN, W. G., and COX, G. M. (1950).—"Experimental Designs." (John Wiley & Sons Inc.: New York.)

FISHER, R. A. (1949).—"The Design of Experiments." (Oliver and Boyd: Edinburgh.)

FONG, J., SCHAFFER, F. L., and KIRK, P. L. (1953).—*Arch. Biochem.* **45**: 319.

GOOD, C. A., KRAMER, H., and SOMOGYI, M. (1933).—*J. Biol. Chem.* **100**: 485.

HILL, C. L., HAWKINS, K. I., and STRASSER, J. P. (1953).—*Fed. Proc.* **12**: 217.

KEMP, A., and VAN HEIJNINGEN, A. J. M. K. (1954).—*Biochem. J.* **56**: 646.

VAN DER KLEIJ, B. J. (1951).—*Biochim. Biophys. Acta* **7**: 481.

LITTLE, J. M. (1949).—*J. Biol. Chem.* **180**: 747.

SATTLER, L., and ZERBAN, F. W. (1950).—*J. Amer. Chem. Soc.* **72**: 3814.

SEIFTER, S., DAYTON, S., NOVIC, B., and MUNTWYLER, E. (1950).—*Arch. Biochem.* **25**: 191.

SHELTAR, M. R. (1952).—*Anal. Chem.* **24**: 1844.

VAN DER VIES, J. (1954).—*Biochem. J.* **57**: 410.

WALAAS, O. (1952*a*).—*Acta Endocr., Copenhagen* **10**: 175.

WALAAS, O. (1952*b*).—*Acta Endocr., Copenhagen* **10**: 193.

WALAAS, O., and WALAAS, E. (1950).—*J. Biol. Chem.* **167**: 769.

YOUNG, M. K. JNR., and RAISZ, L. G. (1952).—*Proc. Soc. Exp. Biol., N.Y.* **80**: 771.

ZONDEK, B., and SHAPIRO, B. (1942).—*Amer. J. Obstet. Gynec.* **44**: 345.

GLYCOGEN, WET WEIGHT, AND DRY WEIGHT CHANGES IN THE VAGINA OF THE MOUSE

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Summary

(i) The subcutaneous or intravaginal administration of oestrone to the ovariectomized mouse causes an increase in the dry weight of the vagina with a maximum at full keratinization. The water content of the tissue is increased in the pre-keratinization phase, and reduced in the keratinization phase. The true glycogen content of the vagina does not increase until keratinization commences.

(ii) The dry weight, wet weight, and total true glycogen content of the vaginae of intact mice in the various stages of the oestrous cycle are compared with those obtained in ovariectomized mice.

(iii) Studies have also been made on the dry weight, wet weight, and total glycogen content of the uterus of intact mice. The pattern of changes seen in this organ is quite different from the pattern of changes seen in the vagina.

(iv) A misleading picture of events is formed if the experimental results are expressed only in the form of ratios of either true glycogen content or wet weight to the dry weight. A full interpretation of the data requires direct reference to the total dry weight, wet weight, or true glycogen content of the organs.

(v) The significance of these findings is discussed in relation to the mode of action of oestrogens and the histochemical aspects of the vaginal response to oestrogens.

I. Introduction

Oestrogens cause rapid proliferation of the epithelium of the vagina of the rodent, the response being characterized by stratification and keratinization. Such changes may be produced in the ovariectomized animal by either systemic or local (intravaginal) injection of oestrogens (see Biggers (1953*a*) for a review of the literature). Few biochemical studies have been made of the vaginal response to oestrogens. The glycogen content of the vagina has been studied because of its relationship to the metabolism of mitosis (Bullough 1952) and to the cellular synthesis of keratin (Scothorne and Scothorne 1953), both phenomena being characteristic of the response of the vagina to oestrogens (Biggers and Claringbold 1954).

The presence of glycogen in the vagina has been demonstrated in man (Miura 1928; Gisbertz 1930; Cruikshank and Sharman 1934; Lison and Vokaer 1949), the monkey (Corner 1923; Miura 1928; van Dyke and Ch'en 1936*a*, 1936*b*), the guinea pig (Tribby 1943), and the mouse (Biggers 1953*a*). Also Raynaud (1941) has described the deposition of glycogen in the urogenital sinus of the mouse embryo. However, glycogen has been reported absent from the vagina of the pig, lamb, and squirrel (Bremicker 1927), and the cat, rat, dog, rabbit, and guinea pig (Miura 1928). All of these workers used histochemical techniques, and it seems that no quantitative chemical studies have been made. Several workers have reported cyclical changes in

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the glycogen of the vagina which may be correlated with the oestrous or menstrual cycles (Miura 1928; Tribby 1943; Lison and Vokaer 1949; Biggers 1953*a*). In studies of the action of oestrone on the vagina of the ovariectomized mouse, Biggers (1953*a*) reported the deposition of glycogen in the stratum spinosum after keratinization had begun.

Few studies have been made on the water content of the vagina. Van Dyke and Ch'en (1936*a*, 1936*b*) demonstrated changes in the monkey during the menstrual cycle. Zuckerman, Palmer, and Hanson (1950) studied the water content in the rat, but their results, although suggestive of changes, were not statistically significant.

In the work to be described below, the glycogen content, wet weight, and dry weight of the vagina of the mouse have been determined simultaneously. Both ovariectomized mice stimulated with oestrone and intact mice have been used.

II. Material and Methods

(*a*) *Animals*

A randomly-bred strain of albino mice was used in this work. In the ovariectomized animal, the response of the vagina was studied, while in the intact animal both the vagina and uterus were studied. The organs were removed as described by Balmain, Biggers, and Claringbold (1956). The vagina was cut into approximately equal parts by dividing it along the line of the urethra; in the case of the uterus each horn was treated as a half-organ.

Each half-organ was allotted at random to one of two determinations using tables of random numbers (Fisher and Yates 1953). In experiment 1 the two determinations were total glycogen and dry weight, while in experiment 2 they were total glycogen and glycogen "blank". Wet weight determinations were made on all pieces of tissue. Twice the mean value obtained in each determination thus yields a mean estimate for the whole organ.

(*b*) *Glycogen Determinations*

The total glycogen and glycogen blank were determined by the methods described by Balmain, Biggers, and Claringbold (1956). The total glycogen represents all the anthrone-positive material present after precipitation with 60 per cent. ethanol and extraction of the precipitate with 5 per cent. trichloroacetic acid (TCA), and consists of the glycogen blank (non-glycogen substances which are extracted and are anthrone-positive), as well as the true glycogen.

(*c*) *Wet and Dry Weights*

The pieces of tissue were mopped roughly dry on filter paper, and weighed quickly on a torsion balance to 0·5 mg. For the dry weight determination the pieces were placed on weighed coverslips, left overnight in an oven at 100°C, cooled in a desiccator, and weighed on a microbalance to 0·1 mg. Observations on 100 vaginae, dissected out and weighed under these conditions, indicated that most of the water was driven off in the first 3 hr of drying. When the drying was continued for a further 21 hr less than 0·5 per cent. further change in the dry weight was found.

(*d*) *Administration of Oestrone to Ovariectomized Mice*

Groups of up to 70 ovariectomized mice were primed 1-6 weeks before use in an experiment by the subcutaneous injection of 1 μg oestrone in nut oil. Five animals were kept per box. One-half of the animals were stimulated by the intravaginal administration of 0·002 μg oestrone in 0·02 ml of 0·01 per cent. aqueous bovine plasma albumin (Biggers 1953*b*), and the other half were stimulated by the subcutaneous administration of 0·5 μg oestrone in 0·1 ml nut oil (Emmens 1950). In both cases the total dose was administered in two injections 24 hr apart. Both of these procedures are expected to elicit 100 per cent. responses within 96 hr, and are thus maximal doses.

Table 1

WET WEIGHT, DRY WEIGHT, AND GLYCOGEN CONTENT OF THE VAGINA OF THE OVARIECTOMIZED MOUSE FOLLOWING OESTROGENIC STIMULATION

Experiment No.	Route of Administration	Measurement	Time after First Injection (hr)					
			0	24	48	72	96	120
1	Intravaginal	Wet weight (mg)	16	27	34	32	30	—
		Dry weight (mg)	3·6	6·0	8·2	7·8	6·4	—
		Total glycogen (μg)	18	32	32	52	52	—
	Subcutaneous	Wet weight (mg)	19	27	40	44	40	—
		Dry weight (mg)	4·2	5·8	9·0	11·0	9·6	—
		Total glycogen (μg)	27	38	28	34	58	—
2	Intravaginal	Wet weight (mg)	20	26	36	30	34	22
		Total glycogen (μg)	44	46	46	74	96	72
		Glycogen blank (μg)	28	24	32	42	40	38
	Subcutaneous	Wet weight (mg)	16	27	44	41	30	31
		Total glycogen (μg)	35	37	41	56	73	54
		Glycogen blank (μg)	26	26	23	25	36	26

(*e*) *Intact Animals*

On the morning of each day on which analyses were made, vaginal smears were used to indicate the stage of the oestrous cycle for each of 100 adult female mice. The smears were stained for 8 min in 1 per cent. methylene blue, and dried in air. Four stages of the oestrous cycle were recognized—dioestrus, pro-oestrus, oestrus, and metoestrus—and these were identified following the original description of Allen (1922). Five animals from each stage were used on each day making 20 in all. This experimental unit will be called a replicate.

III. Results

Since the data are extensive (involving approximately 2500 observations) only mean values will be tabulated, and the data reduced by means of the analysis of variance and covariance (Fisher 1948). Table 1 gives the mean values for ovariectomized animals following oestrogenic stimulation, and Tables 2 and 3 the corresponding analyses of variance. Table 4 gives the mean values for intact animals. Preliminary examination of the data showed that it was reasonable to assume that the observations were log-normally distributed. Consequently, all analyses have been made by using logarithms, and the mean values shown in Tables 1 and 4 are obtained by taking antilogarithms. Since logarithms have been used, standard errors cannot be given on an arithmetic scale (cf. Cochran and Cox 1950; Hald 1952, for discussion of the transformation). In this case an appropriate measure of accuracy of a mean value are the 5 per cent. fiducial limits, and these are given in Table 4. They are not given in Table 1, since estimates of error are available from the analysis of variance tables.

Table 2

ANALYSES OF VARIANCE FOR THE DATA OBTAINED IN EXPERIMENT 1

The degrees of freedom corresponding to times have been partitioned by orthogonal polynomial coefficients as described by Fisher and Yates (1953)

Source of Variation	D.F.		Mean Squares		
			Wet Weight	Dry Weight	Glycogen
Replicates (A)	2		6·5*	4·5*	54·6**
Routes of administration (B)	1		60·9***	29·4***	1·5
Times (C)	(4)				
Linear		1	429·4***	229·8***	275·6***
Quadratic		1	178·4***	101·2***	1·1
Cubic		1	0·2	3·3	20·1**
Quartic		1	2·7	1·6	30·9***
First order interactions					
A×B	2		0·3	1·8	0·1
A×C	8		3·1	2·2	4·5
B×C	4		4·6*	5·9**	16·8***
Second order interactions					
A×B×C	8		1·2	0·9	0·8
Error	150		1·81	1·16	2·65

* 0·05 > P > 0·01.
** 0·01 > P > 0·001.
*** P < 0·001.

(*a*) *Ovariectomized Mice*

(i) *Wet Weight*.—The analyses of variance (Table 2) show that in both experiments large changes in wet weight occurred in the vagina after the administration of oestrone by both subcutaneous and intravaginal routes. In both analyses there was a significant interaction between the effects of route of administration and the time after the first injection, showing that the changes are different after each route of administration. Examination of the mean values shows that subcutaneous injection produced a greater increase in wet weight than intravaginal injection. In the first experiment, maximum wet weight was reached 48 hr after intravaginal administration and 72 hr after subcutaneous administration, after which the wet weight decreased.

In the second experiment, the maximum wet weight was the same for both routes and was reached between 48 and 72 hr; the significant quintic mean square is due to fluctuations in the falling of wet weight, of unknown cause. The exact description of this phase must await further investigation.

(ii) *Dry Weight.*—The analysis of variance (Table 2) indicates that dry weight changes are of a similar form to wet weight changes, although the mean values show that the changes are not proportional. Subcutaneous injection produced a greater increase in dry weight than intravaginal injection.

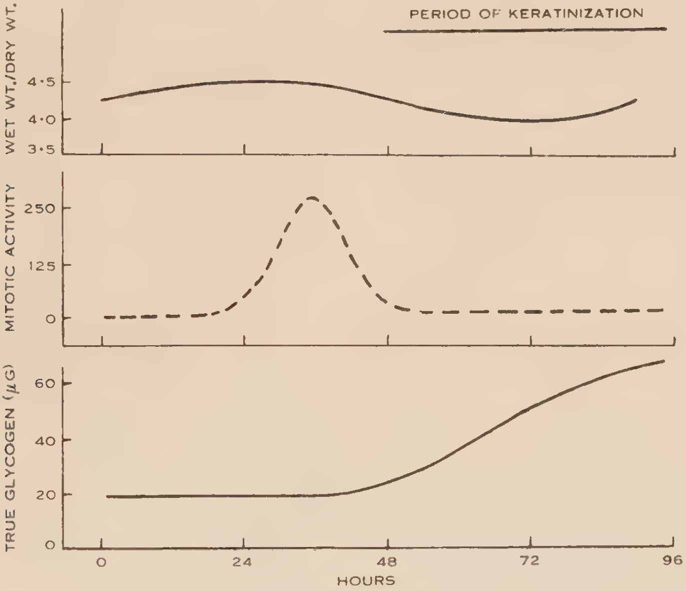

Fig. 1.—Schematic account of the variables studied against the same time-axis. In addition, the mitotic activity (taken from Biggers and Claringbold 1954) is indicated. The period of keratinization was determined by Biggers (1953*a*).

A set of readings of wet weight and dry weight from the same half-vagina was available for further analysis. The difference between the logarithms of the wet and dry weights is the logarithm of the wet weight/dry weight ratio. Since the two measurements are highly correlated ($r_{(149)} = 0{\cdot}892$, $P < 0{\cdot}001$) an analysis of covariance was made. From this analysis a common estimate of the variances of the log wet weight/dry weight ratio was computed. This estimate was used to test the homogeneity of the ratios obtained on different days, and between different routes of administration. The test of significance showed extremely highly significant changes in the ratio from day to day ($\chi^2_{(4)} = 34{\cdot}0$, $P < 0{\cdot}001$), there being no significant difference between routes. The result is shown graphically in Figure 1. It can be seen that the water content of the vagina was raised above the control level over the first 48 hr and was lowered in the subsequent 48 hr.

(iii) *Total Glycogen.*—The analyses of variance (Table 3) for both experiments indicate highly significant differences in the glycogen content of the vagina at different times after the injection of oestrone. In both analyses, a significant routes ×times interaction shows that the pattern of change of total glycogen depends on the route of administration. When subcutaneous administration was used there was a sudden rise which commenced between 48 and 72 hr, reached a peak at 96 hr, and then fell off. After intravaginal administration this rise was slightly in advance of that observed with subcutaneous administration. Although the curves follow different patterns in the later stages, the results clearly demonstrate a large rise in total glycogen after 48 hr from the first injection in both cases.

TABLE 3

ANALYSES OF VARIANCE OF THE DATA OBTAINED IN EXPERIMENT 2

Source of Variation	D.F.	Mean Square		
		Total Glycogen	Glycogen Blank	Wet Weight
Replicates (A)	1	30·9***	28·0***	0·5
Routes of administration (B)	1	2·4	4·0	22·2***
Times (C)	(5)			
Linear	1	123·0***	26·3***	106·9***
Quadratic	1	4·1*	0·1	247·0***
Cubic	1	36·0***	18·5***	0·4
Quartic	1	8·8**	0·9	0·9
Quintic	1	1·6	0·0	7·7***
First order interactions				
A×B	1	3·1	1·7	1·9
A×C	5	1·2	4·3	2·4
B×C	5	3·7*	0·4	5·3**
Second order interactions				
A×B×C	5	0·0	2·0	1·9
Error	96	0·82	1·32	1·32

* $0{\cdot}05 > P > 0{\cdot}01$.

** $0{\cdot}01 > P > 0{\cdot}001$.

***$P < 0{\cdot}001$.

(iv) *Glycogen Blank.*—The mean values and the analysis of variance (Table 3) show that a significant rise in the blank occurred corresponding to the rise in total glycogen, and that no differences were observed between routes of administration.

The difference between the logarithms of total glycogen and glycogen blank is a measure of the amount of true glycogen, and this was computed from the data of experiment 2. The variance of this difference must take account of any correlation of these two measurements, since they are made on the one organ. The correlation coefficient is $r_{(95)} = -0{\cdot}04$, $P>0{\cdot}05$. From the analyses of variance and covariance, the variance of the difference was estimated for each time and route of administration. Subsequent analysis, as used in analysing the wet weight/dry weight ratio, indicates that only the time differences are significant ($\chi^2_{(4)} = 21{\cdot}4$, $P<0{\cdot}001$). The result is shown graphically in Figure 1, where it is seen that there was a considerable increase in the true glycogen 48 hr after the first injection.

(b) *Intact Mice*

Preliminary examination of the data indicated that the variance of the measurements depends on the stage of the oestrous cycle. Therefore, individual analyses were made within each stage, after removing sums of squares attributable to differences between replicates. From the error term in each analysis the 5 per cent. fiducial limits for the mean values were calculated (Table 4).

TABLE 4

WET WEIGHT, DRY WEIGHT, AND GLYCOGEN CONTENT OF THE VAGINA OF THE MOUSE AT VARIOUS STAGES OF THE OESTROUS CYCLE. MEANS AND 5% FIDUCIAL LIMITS ARE TABULATED

Experiment No.	Stage of Oestrous Cycle	Vagina		Uterus	
		Wet Weight (mg)	Dry Weight (mg)	Wet Weight (mg)	Dry Weight (mg)
1	Dioestrus	27·1 (25·1-29·2)	6·08 (5·56-6·65)	26·4 (21·8-31·8)	5·50 (4·57-6·61)
	Pro-oestrus	64·9 (60·4-69·7)	15·7 (14·5-16·9)	112 (96·4-130)	19·3 (17·0-22·0)
	Oestrus	55·6 (51·1-60·5)	13·5 (12·2-15·0)	70·7 (63·5-78·5)	13·5 (12·3-14·7)
	Metoestrus	32·7 (30·3-35·4)	7·02 (6·41-7·68)	39·9 (34·8-45·7)	8·18 (7·30-9·19)
		Total Glycogen (μg)	Glycogen Blank (μg)	Total Glycogen (μg)	Glycogen Blank (μg)
2	Dioestrus	52·1 (47·0-57·8)	23·0 (21·3-24·9)	29·5 (23·9-36·5)	22·4 (18·0-27·9)
	Pro-oestrus	46·5 (40·2-53·7)	20·6 (17·9-23·7)	94·9 (76·2-118)	33·7 (30·1-37·8)
	Oestrus	41·3 (36·8-46·3)	24·2 (21·5-27·2)	85·3 (75·5-96·4)	39·9 (38·2-41·7)
	Metoestrus	62·5 (52·5-74·5)	30·5 (27·5-33·8)	50·9 (44·5-58·4)	36·7 (31·9-42·3)

(i) *Wet Weight.*—In both the vagina and uterus, the wet weight changes were parallel, with a maximum at pro-oestrus, but the changes in the uterus were very much greater.

(ii) *Dry Weight.*—Dry weight changes were similar to the wet weight changes for both organs. The wet weight/dry weight ratio was calculated for each stage in each organ, and is illustrated graphically in Figure 2, where two differences between each organ may be seen. First, the uterus contained more water at all stages than the vagina. Secondly, at the pro-oestrous and oestrous stages the vagina was dehydrated and the uterus hydrated, relative to the metoestrous and dioestrous stages.

(iii) *Total Glycogen.*—The changes in total glycogen were entirely different in the two organs. In the vagina the minimum level was at oestrus and the maximum was at metoestrus. In the uterus total glycogen was minimal at dioestrus and maximal at pro-oestrus.

(iv) *Glycogen Blank.*—These changes were similar to the changes of total glycogen although smaller in degree. The differences between total glycogen and glycogen blank, i.e. true glycogen, have been plotted in Figure 3 for both organs, where large differences in the distribution of true glycogen are seen. In the vagina the maximum amount of glycogen is present at metoestrus and the minimum amount at oestrus, whilst in the uterus the maximum amount is at pro-oestrus and the minimum at dioestrus.

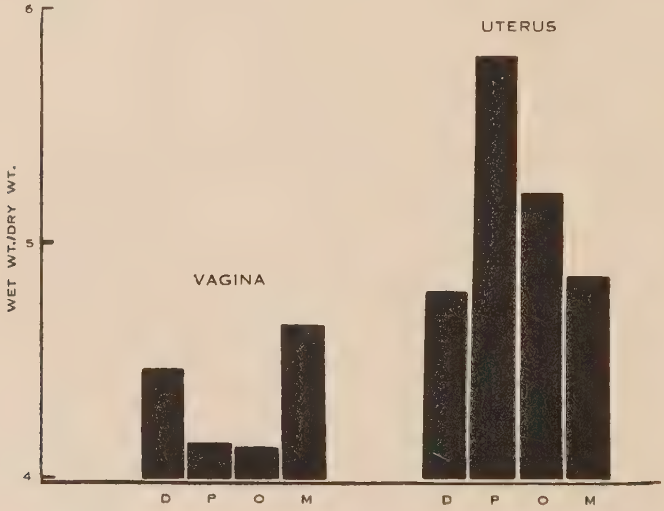

Fig. 2.—Comparison of wet weight/dry weight of the vagina and uterus at the four stages of the oestrous cycle. *D*, Dioestrus; *P*, pro-oestrus; *O*, oestrus; *M*, metoestrus. Whilst at every stage the uterus is more saturated than the vagina, the relative pattern of change is quite different.

IV. Discussion

(*a*) *Growth and Water Content*

The results show that both subcutaneous and intravaginal injection of oestrone cause growth of the vagina, manifested by an increase in dry weight. Maximum growth is attained at the time of full keratinization, and is reached earlier with the intravaginal route of administration. The wet weight changes in the organ do not follow proportionately the dry weight changes, and this fact implies that differential shifts in water occur during the response (Fig. 1). The water content is increased in the early pre-keratinization phase, the period of high mitotic activity and active growth (Allen, Smith, and Gardner 1937; Biggers and Claringbold 1954) while after keratinization the tissue is relatively dry in comparison with the resting organ. These results do not agree with those described by Zuckerman, Palmer, and Hanson (1950), who studied the ovariectomized rat following the subcutaneous injection of oestradiol-3,17β. In all of our experiments significant secular changes occurred in all variables (Tables 2 and 3), and were also observed in the studies on intact mice. These large shifts make it imperative that all comparisons be made simultaneously. This requirement was not fulfilled in the work of Zuckerman *et al.*, and may thus account for the non-significance of their findings. It should also be noted that these changes are different from the changes in the uterus following oestrogen administration (see Reynolds (1949) for a review of the literature).

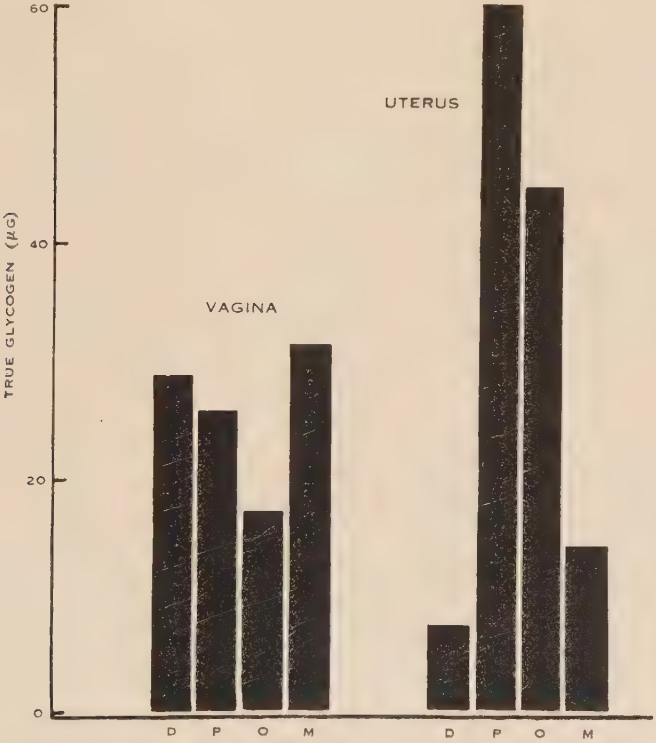

Fig. 3.—True glycogen content of the uterus and vagina at the four stages of the oestrous cycle. *D*, Dioestrus; *P*, pro-oestrus; *O*, oestrus; *M*, metoestrus. The pattern of change in the uterus is quite different to that seen in the vagina.

In intact animals there is a striking difference in water content between the uterus and vagina (Fig. 2). In the vagina the changes are similar to those following artificial stimulation by either intravaginal or subcutaneous administration. In both cases reduced water content is associated with the process of keratinization. In the uterus there is a large increase in water content at the time of vaginal cornification, and this is associated with the distension of the uterus with intra-uterine fluid at this stage.

The results described above show that the vaginal response, as measured by wet weight or dry weight, was larger after subcutaneous administration than after the intravaginal administration. This may have been due to non-equivalence of the subcutaneous and intravaginal doses. As the relevant dose-response lines are unknown, this point cannot as yet be decided. However, Claringbold and Biggers (1955) have shown that fundamental differences exist in the action of oestrone given by both routes of administration, possibly due to changes in the connective tissue beneath the vaginal epithelium. The large increase in wet and dry weight, after subcutaneous administration, may well be due to connective tissue changes which are not elicited by the intravaginal route of administration.

(*b*) *Glycogen Content*

Since there are changes in wet and dry weight, the observations on glycogen content have not been placed on a wet weight or dry weight basis; to do so automatically—a very frequent practice—would obscure the phenomena which take place. Instead, the figures refer to absolute amounts in the whole organ. Such considerations apply to any biochemical measurements made on this organ.

The results show that glycogen is formed in considerable amounts in the vagina after keratinization has commenced, this being observed in intact mice undergoing normal oestrous cycles and also in ovariectomized mice artificially stimulated with oestrone. In the control animals, and in those in which the early stage of the response had been experimentally induced, only small amounts of glycogen were present. These changes have been studied histochemically in the mouse by Biggers (1953*a*) using the periodic acid-Schiff technique. In the early stages no reaction was obtained in the stratum spinosum, but after keratinization had commenced, considerable amounts of glycogen were detected in this region of the epithelium. Thus the histochemical results are consonant with the more sensitive biochemical results. The findings, however, seem to be disconsonant with the histochemical studies of Tribby (1943) who, working with guinea pigs, described the deposition of glycogen in the stratum spinosum during pro-oestrus. Since the work of Tribby was done on intact animals it is difficult to decide whether this finding indicates a species difference or is due to difficulties in the exact diagnosis of the stages of the oestrous cycle. For example, in the stage designated pro-oestrous by Tribby, keratinization is well developed, and therefore the differences between his and our results may be illusory. This problem of the diagnosis of the stages of the oestrous cycle is only avoided by the artificial production of cornification.

The observations reported above show that completely different phenomena occur in the vagina and in the uterus during oestrogenic stimulation, and show that each organ responds in a characteristic manner to the hormone. Cognisance of this fact should be taken in studies on the action of oestrogens or other hormones, since it challenges the validity of generalizations from one organ to another.

The presence of glycogen in growing and keratinizing epidermis and its derivatives is a widespread phenomenon, e.g. foetal epidermis (Bernard 1859), healing wounds (Bradfield 1951), skin autografts (Scothorne and Scothorne 1953), and hair follicles (Hardy 1953). In several papers Bullough and his colleagues have referred to unpublished work by Bullough and Eisa on the glycogen content of the skin of the female mouse following the injection of oestrone. They claim that the high glycogen level coincides with maximum mitosis. The same workers have also mentioned the deposition of glycogen in the vagina and uterus of the mouse during the oestrous cycle, and have described in the vagina a biphasic rise in glycogen and lactic acid. Recently several workers have speculated on the function of the glycogen in epithelial tissues (Bullough 1950, 1952; Bullough and van Bordt 1950; Bradfield 1951; Carruthers and Suntzeff 1953; Scothorne and Scothorne 1953), and it has been suggested that the glycogen is concerned with the metabolism of mitosis and also with the synthesis of keratin.

Our work has shown that the period of accumulation of glycogen in the vagina does not coincide with the period of great mitotic activity, but that it appears after most cell divisions are complete. Thus the suggestion of Bullough (1952) that the mobilization of glycogen stimulates cell division, is not supported by our work. The appearance of glycogen in the vagina coincides with the formation of keratin, and Biggers (1953*a*) showed histochemically that the glycogen is in the stratum spinosum just beneath the region undergoing keratinization. As yet its function remains obscure.

V. Acknowledgments

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VI. References

Allen, E. (1922).—*Amer. J. Anat.* **30**: 297.

Allen, E., Smith, G. M., and Gardner, W. U. (1937).—*Amer. J. Anat.* **61**: 321.

Balmain, J. H., Biggers, J. D., and Claringbold, P. J. (1956).—*Aust. J. Biol. Sci.* **9**: 139.

Bernard, C. (1859).—*C.R. Acad. Sci., Paris* **48**: 673.

Biggers, J. D. (1953*a*).—*J. Anat., Lond.* **87**: 327.

Biggers, J. D. (1953*b*).—*J. Endocrinol.* **9**: 136.

Biggers, J. D., and Claringbold, F. J. (1954).—*J. Anat., Lond.* **89**: 124.

Bradfield, J. R. G. (1951).—*Nature* **167**: 40.

Bremicker, W. (1927).—*Arch. Gynaek.* **129**: 807.

Bullough, W. S. (1950).—*Brit. J. Cancer* **4**: 329.

Bullough, W. S. (1952).—*Biol. Rev.* **27**: 133.

Bullough, W. S., and van Bordt, G. J. (1950).—*Acta Endocr. Copenhagen* **4**: 291.

Carruthers, C., and Suntzeff, V. (1953).—*Physiol. Rev.* **33**: 229.

Claringbold, P. J., and Biggers, J. D. (1955).—*Aust. J. Biol. Sci.* **8**: 407.

Cochran, W. G., and Cox, G. M. (1950).—"Experimental Designs." (John Wiley & Sons Inc.: New York.)

Corner, G. W. (1923).—*Contr. Embryol. Carneg. Instn.* **15**: 73.

Cruikshank, R., and Sharman, A. (1934).—*J. Obstet. Gynaec. Brit. Emp.* **41**: 190.

van Dyke, H. B., and Ch'en, G. (1936*a*).—*Amer. J. Anat.* **58**: 473.

van Dyke, H. B., and Ch'en, G. (1936*b*).—*Ergebn. Physiol.* **38**: 836.

Emmens, C. W. (1950).—"Hormone Assay." Ch. 13. (Academic Press Inc.: New York.)

Fisher, R. A. (1948).—"Statistical Methods for Research Workers." 10th Ed. (Oliver and Boyd: Edinburgh.)

Fisher, R. A., and Yates, F. (1953).—"Statistical Tables for Biological, Agricultural and Medical Research. 4th Ed. (Oliver and Boyd: Edinburgh.)

Gisbertz, P. W. (1930).—*Mschr. Geburtsh. Gynäk.* **84**: 24.

Hald, A. (1952).—"Statistical Theory with Engineering Applications." (John Wiley & Sons Inc.: New York.)

HARDY, M. H. (1952).—*Amer. J. Anat.* **90**: 285.

LISON, L., and VOKAER, R. (1949).—*Ann. Endocr., Paris* **10**: 66.

MIURA, H. (1928).—*Mitt. med. Akad. Kioto.* **2**: 1.

RAYNAUD, A. (1941).—*C.R. Soc. Biol., Paris* **135**: 491.

REYNOLDS, S. R. M. (1949).—"Physiology of the Uterus." 2nd Ed. (Paul B. Hoeber Inc.: New York.)

SCOTHORNE, R. J., and SCOTHORNE, A. W. (1953).—*J. Anat., Lond.* **87**: 11.

TRIBBY, C. L. (1943).—*Anat. Rec.* **86**: 425.

VOKAER, R. (1952).—*Ann. Endocr., Paris* **13**: 500.

ZUCKERMAN, S., PALMER, A., and HANSON, D. A. (1950).—*J. Endocrinol.* **6**: 261.

FUNGAL CELLULASES

VII. STACHYBOTRYS ATRA: PRODUCTION AND PROPERTIES OF THE CELLULOLYTIC ENZYME

By Robert Thomas*

[*Manuscript received June 21, 1955*]

Summary

Stachybotrys atra grows well on a Waksman-Carey medium containing cellulose as the sole carbon source; an extracellular cellulase is produced which is probably adaptive. The conditions conducive to a high yield of enzyme have been investigated and details are given of a relatively simple bulk-culture apparatus.

Considerable attention has been devoted to the cellulase assay procedure based on changes in viscosity of soluble cellulose derivatives. A new assay method, involving the change in birefringence of "Cellophane" during the initial stages of hydrolysis, has been investigated.

A procedure is described for concentrating the enzyme to a salt-free freeze-dried powder, with negligible loss of activity; the general properties of this preparation have been studied. The powder consists of approximately equal amounts of protein and carbohydrate, the latter on acid hydrolysis yielding galactose, mannose, glucose, and some xylose. Paper electrophoresis indicates the presence of at least three proteins, probably only one of which is cellulolytic and which is apparently intimately associated with the carbohydrate moiety.

The cellulase is active against a considerable range of β-1, 4′-polyglucosides extending from filter paper to cellotriose, but is without effect on highly crystalline cellulose (cotton duck) or cellobiose. It will hydrolyse xylan but not chitin. The terminal products of the enzymic degradation of cellulose were identified as cellobiose and glucose and the mode of formation of these sugars is discussed.

I. Introduction

In a previous paper of this series, Jermyn (1953) described the growth and enzyme production of *Stachybotrys atra* Corda on non-cellulosic substances. The present paper describes the culture of the same organism on a medium containing cellulose as the sole carbon source. Under these conditions an extracellular cellulase is produced which occurs only in very low concentrations in non-cellulosic culture filtrates.

II. Growth Conditions

(*a*) *Culture Methods*

In an examination of the optimum conditions for cellulase production by *S. atra*, the organism was grown in submerged culture at 28°C in 250-ml conical flasks, each plugged with cotton wool and containing 50 or 100 ml of medium. Other details of the cultural conditions, including the composition of the modified Waksman-Carey medium, have been described by Jermyn (1953). The carbon source consisted of an acid-treated cotton cellulose (Whatman cellulose powder, ashless grade) at a concentration of 10 g/l.

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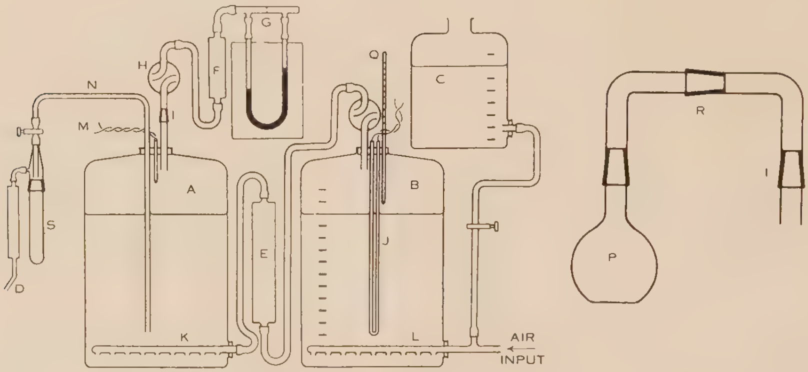
A bulk-culture apparatus (Figs. 1 (*a*) and 1 (*b*)) was constructed from relatively simple components. The fermentation vessel *A* consisted of a 15-l. "Pyrex" aspirator containing 10 l. of medium. Aeration was effected by means of a perforated glass tube *K*; the resulting agitation was adequate to keep the medium well mixed. The input and output filters (*E* and *F* respectively) were packed with non-absorbent cotton wool and the airflow rate (approx. 13 l/min) measured by the pressure drop across a sharp-edged orifice *G*, using a water manometer previously calibrated against a standard rotameter. The apparatus was operated in a constant temperature room at 28°C and saturated air at this temperature passed into the input filter *E* from an aspirator *B* containing water. A heating element *F* compensated for the temperature drop in *B* due to latent heat of evaporation losses; the effluent air temperature was measured by a thermometer *Q* inserted in the neck of the aspirator. The aspirator *C* served as a reservoir by means of which the level of water in *B* was readjusted as required.



(a) (b)

Fig. 1.—(*a*) Bulk-culture apparatus: *A*, Fermentation bottle; *B*, bottle for saturating air entering *A*; *C*, water reservoir for *B*; *D*, sampling device; *E*, input filter; *F*, output filter; *G*, sharp-edged orifice attached to water manometer; *H*, splash head; *J*, heating element; *K*, *L*, aeration tubes; *M*, thermistor; *N*, glass tube leading to sampling tube *S*; *Q*, thermometer. (*b*) *I*, Inoculating orifice; *P*, inoculating flask; *R*, ground-glass joint.

The sampling device *D* consisted of a glass tube *N* connected by a short length of rubber tubing to a ground-glass joint assembly and filter, such that a sample of the culture could be drawn over into the tube *S*, which could then be replaced by a fresh sterile tube. The primary inoculum consisted of a sporing culture grown on potato-dextrose agar. This was used to inoculate a 100-ml quantity of the cellulose medium contained in a 500-ml ground-glass-jointed flask (*P*, Fig. 1(*b*)). After 8 days growth at 28°C on a reciprocating shaker, this culture was used to inoculate the bulk culture. This was effected by means of two right-angled, ground-glass joints, one end of which was attached to the inoculating flask and the other to the inoculating orifice *I* as shown in Figure 1 (*b*), observing normal sterile precautions throughout. The inoculum was transferred by rotating the flask *P* through 180° about the joint *R*. The sterile splash head H, attached to the output filter F, was then substituted for the inoculating device at the joint I and aeration commenced.

Normally, two fermentation bottles were connected in parallel to the saturated air supply from bottle B thus yielding 20 l. of culture filtrate from each run. Frothing during the fermentation was only moderate and did not necessitate the addition of an antifoaming agent. The fermentation temperature, as measured by a thermistor M inserted in the neck of the culture vessel A, varied less than 0·5°C during the normal 10- to 12-day growth period.

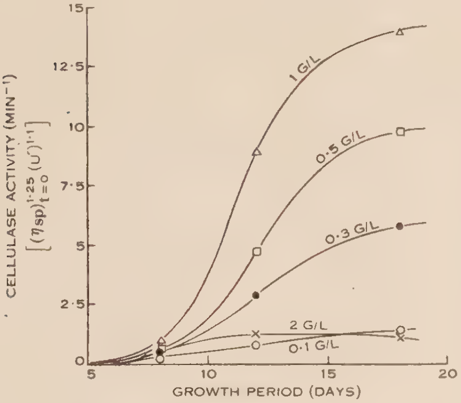


Fig. 2

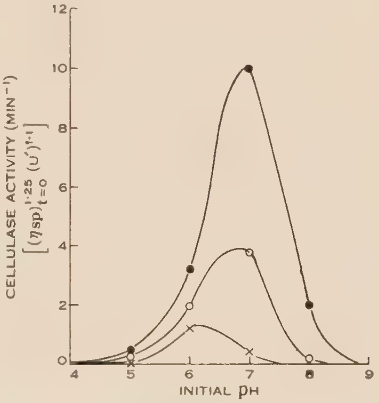


Fig. 3

Fig. 2.—Cellulase yield from Waksman-Carey media containing cellulose at various concentrations; shake flasks incubated at 28°C. Activity assayed viscometrically against SCMC at pH 5·0 and 30°C.

Fig. 3.—Influence of initial pH of Waksman-Carey media (1 per cent. cellulose) on cellulase production in shake flask culture at 28°C; initial pH adjusted with N HCl or N NaOH and redetermined after autoclaving. Activity assayed viscometrically against SCMC at pH 5·0 and 30°C: × 4 days, ○ 7 days, ● 13 days growth respectively.

(*b*) *Optimum Conditions for Cellulase Production*

Except where otherwise stated, these investigations were carried out in 250-ml flasks by the shake-culture method; the results given are those of combined samples from duplicate flasks. Cellulase activities were assayed by a viscometric method (see Section III) in which the substrates were either sodium carboxymethylcellulose (SCMC), or "Modocoll M", an ethyl hydroxyethylcellulose (EHEC).

The influence of the concentration of cellulose in the culture medium was investigated and the results are shown in Figure 2. The existence of an optimum cellulose concentration (*c.* 1 g/l) is probably a consequence of the dependence of mycelial growth and cellulase production on the availability of substrate up to a limiting concentration, coupled with the experimentally observed adsorption of cellulase on cellulose (described below).

S. atra, when grown on a medium containing glucose as the sole source of carbon produces an extracellular *p*-nitrophenyl-β-glucosidase (Jermyn 1953). A 3-day culture grown on 1 per cent. glucose medium was examined for cellulase activity;

0·27 per cent. of the glucose had been consumed, and, although the filtrate readily hydrolysed *p*-nitrophenyl-β-glucoside, no cellulase activity could be detected. After a 26-fold concentration in a vacuum evaporator at 28°C, the dialysed concentrate showed a slight trace of cellulase activity. Thus the output of cellulase is considerably increased when cellulose is present in the medium and in this respect it behaves as an adaptive enzyme. It was also observed that filtrates from the cellulose medium did not possess any *p*-nitrophenyl-β-glucosidase activity.

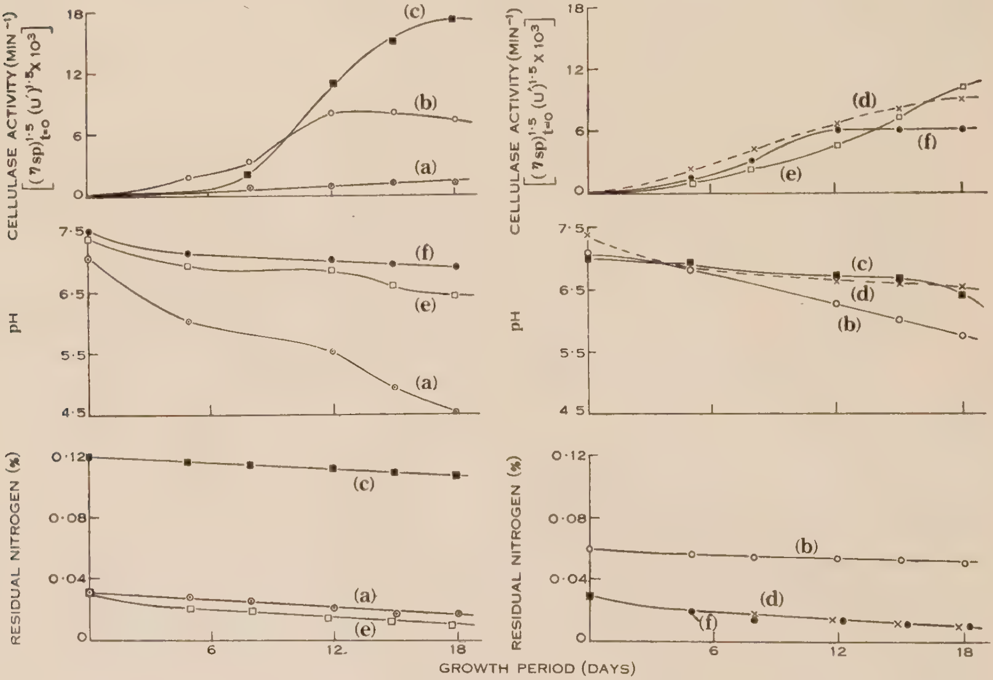


Fig. 4.—Influence of buffering capacity of the medium on growth and cellulase production. Phosphate and nitrogen supplied as: (*a*) $(NH_4)_2HPO_4$, 0·6 g/l; (*b*) $(NH_4)_2HPO_4$, 2·5 g/l; (*c*) $(NH_4)_2HPO_4$, 5·0 g/l; (*d*) $(NH_4)_2HPO_4$, 0·6 g/l; Na_2HPO_4, 2·0 g/l; (*e*) $(NH_4)_2HPO_4$, 0·6 g/l; K_2HPO_4, 2·0 g/l; (*f*) $(NH_4)_2HPO_4$, 0·6 g/l; K_2HPO_4, 4·0 g/l. Cellulase activity assayed viscometrically against EHEC at pH 5·0 and 30°C.

The effect of varying the initial pH of the medium between 4 and 9 was examined by adjusting the pH of the normal medium (i.e. 7·1-7·3) with N HCl or N NaOH and determining the rate of cellulase production (Fig. 3). The results demonstrated the importance of initial pH, since no significant growth or cellulase production occurred outside the pH range 5 to 8. The optimum initial pH corresponded approximately to that of the normal medium.

The major inorganic constituent of the culture medium was $(NH_4)_2HPO_4$ (2·5 g/l), which, in addition to serving as a source of nitrogen and phosphorus, was also responsible for the buffering capacity of the solution. The influence both of varying the concentration of this component and of adding other buffering phosphates at constant $(NH_4)_2HPO_4$ concentration was investigated. The results are set out in Figure 4, including the changes in pH and nitrogen content of the media throughout the growth period. The cellulase activities were assayed by the viscometric method, using EHEC rather than SCMC as the substrate, since the viscosity of SCMC is dependent upon the ionic strength of the solution. The viscosity of the EHEC assay solution was unchanged on adding sodium chloride to a final concentration of 0·6 per cent., i.e. ionic strength = 0·1, although at higher values a gradual precipitation ensued. This salt concentration also had no effect on the rate of depolymerization of the EHEC solution by a dialysed enzyme solution, thus indicating that the cellulase activity is inpedendent of ionic strength within this limited range.

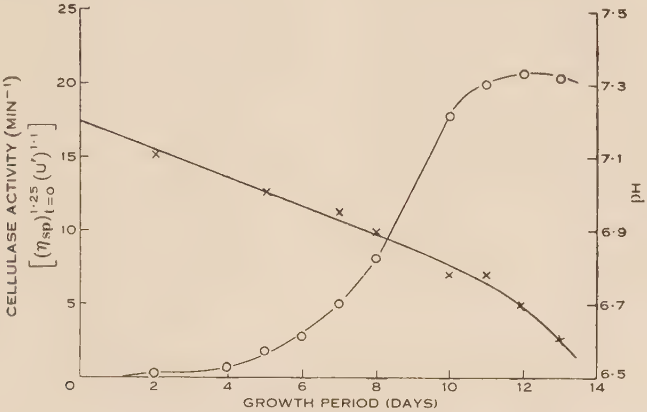


Fig. 5.—Typical cellulase production curve for bulk culture incubated at 28°C. Activity assayed viscometrically at pH 5·0 and 30°C. ○ Enzyme activity; × pH.

It is evident from these results, that the value of a high ammonium phosphate concentration resides in the increased buffering capacity of the medium. This can be seen by comparing the curves (*a*), (*b*), and (*c*) in Figure 4; the low yield of enzyme in (*a*) was clearly not due to exhaustion of the inorganic nitrogen supply and apparently resulted from inadequate buffering (cf. curves (*d*) and (*e*) in Fig. 4).

In a further experiment, enzyme production on media containing double and quadruple concentrations of all the salts other than ammonium phosphate, was compared with that obtained on the standard medium. There was no significant difference during a 20-day growth period. Thus the peak cellulase activity is not limited by the exhaustion of any of the constituents of the medium, unless perhaps the accessible (i.e. amorphous) regions of the cellulose are consumed. A possible limiting factor may be the production of toxic substances, for example some of the products of autolysis of mycelium, which inhibit further growth and cellulase formation.

During the bulk-culture experiments, the rate of aeration was found to influence the cellulase output; an optimum yield was obtained at an airflow rate of approximately 13 l/min, as measured by a standard rotameter at atmospheric pressure. The existence of an optimum aeration rate is understandable, the minimum value being determined by the oxygen requirement and the efficiency of agitation of the culture, while the maximum limiting rate probably depends on the ease of denaturation of the enzyme at the air-water interface. The results of a typical bulk-culture experiment are shown in Figure 5. In general, the peak cellulase activity was obtained in a shorter period than was the case with the shake-culture flasks. Futhermore, the yield was slightly higher and the corresponding pH drop a little less.

To obtain an approximate estimate of the mycelial content of the final mycelium-cellulose residue, the nitrogen content of the washed and dried residue was compared with that of the mycelium from a 3-day culture grown in shake-culture flasks on a medium containing 2 per cent. glucose as the carbon source. This mycelium contained 4·6 per cent. nitrogen, whereas the mycelial residues from the cellulose medium contained 1·1 per cent. nitrogen, or 24 per cent. of the glucose medium nitrogen. Both of these mycelial residues, on extraction with light petroleum, gave a low yield of a colourless crystalline product which was subsequently shown to be ergosterol (Thomas 1955*a*).

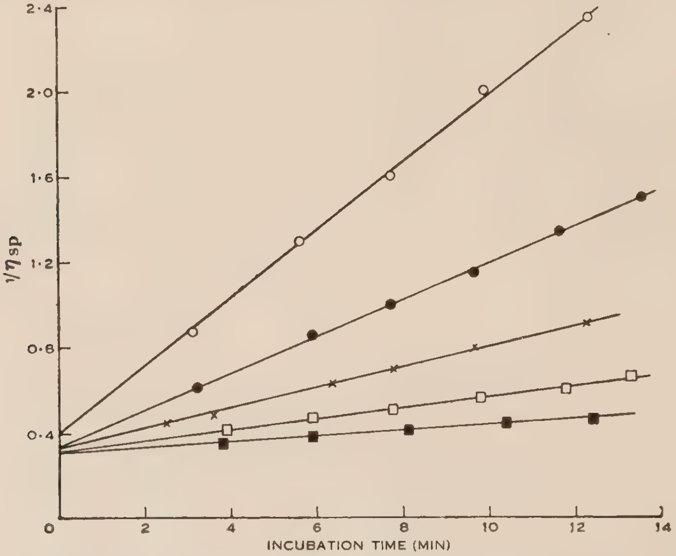


Fig. 6.—Relation between the reciprocal of the specific viscosity η_{sp} of SCMC and time, when incubated with various concentrations of cellulase. The curves correspond to the data given in Table 1 for an initial specific viscosity $(\eta_{sp})_{t=0} = 3{\cdot}60$.

III. Assay Methods

(*a*) *Decrease in Viscosity of Solutions of Cellulose Derivatives*

The method is based on that described by several workers for determining cellulase activity (Levinson and Reese 1950; Tracey 1950; Sandegren and Enebo 1952; Preece and Aitken 1953). The soluble cellulose derivatives used were SCMC* and EHEC.† Stock solutions were prepared by continuously shaking an aqueous suspension of the powder overnight at room temperature, any undissolved residue being removed by filtering the solution through glass wool under reduced pressure. These solutions were usually stable for several weeks when stored at 5°C. The stock solutions of SCMC (1·5 per cent.) or EHEC (1·0 per cent.) were thoroughly mixed by a gentle swirling action, before diluting with water to a suitable viscosity for assay purposes.

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* High viscosity "Cellofas B", Imperial Chemical Industries Ltd.

† "Modocoll M", Mo and Domsjö Ltd., Ornsköldsvik, Sweden.

The assay procedure consisted of adding the enzyme solution (2 ml) to a mixture of solutions of the cellulose derivative (5 ml) and pH 5·0 McIlvaine citrate-phosphate buffer (2 ml). 8 ml of this mixture were then pipetted into a No. 2 B.S.S. Ostwald viscometer (water flow time of 15·0 sec at 30°C) and the flow time observed at frequent intervals during 15 min incubation at 30°C. In general, the stock SCMC or EHEC solutions were diluted to give an initial flow time of approximately 60 sec, i.e. initial specific viscosity $((\eta_{sp})_{t=0}) = 3$. The initial flow time was taken as the value obtained on substituting distilled water for the enzyme solution.

This procedure was used to investigate the relationship between enzyme concentration and the rate of decrease of viscosity of SCMC solutions. It was found that at enzyme concentrations where the rate of breakdown is not too high, there is an approximately linear relation between the reciprocal of specific viscosity and time, i.e.

$$1/\eta_{sp} \propto t, \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where $\eta_{sp} = \eta_r - 1$,
η_{sp} = specific viscosity, and
η_r = relative viscosity.

A series of curves obtained at different enzyme concentrations are shown in Figure 6; electrodialysed enzyme solutions were used in order to minimize errors due to variation in ionic strength.

Hultin (1946, 1949) in a theoretical study of the viscometric assay of enzyme activity, deduced a relationship which may be written as follows:

$$E = C^2 U', \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

where E = enzyme concentration in units per g solution,
C = substrate concentration in g per g solution, and
$U' = \mathrm{d}(1/\eta_{sp})/\mathrm{d}t$.

This predicts a linear relation between E and U' in the presence of excess substrate. In order to test this prediction, U' was determined at five selected enzyme concentrations, using an electrodialysed culture-filtrate preparation diluted with varying amounts of water. The determinations were carried out with three SCMC solutions of differing initial specific viscosity, and the results are given in Table 1.

Clearly, there is an appreciable deviation from linearity. However, if the relation between E and U' is such that

$$E \propto (U')^x, \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

then since

$$x = \frac{\mathrm{d}(\log E)}{\mathrm{d}(\log U')},$$

its value can be calculated from the above data. The results of these calculations are set out in Table 2. Statistical analysis indicated that within the range of enzyme and substrate concentrations shown in Table 1, the value of the parameter x and its standard error is given by $x = 1{\cdot}11 \pm 0{\cdot}01$. A corresponding examination of the suitability of EHEC as an assay substrate (see below) showed that it closely parallels SCMC in its behaviour, although its sensitivity is considerably lower. Here again the relation between E and U' is non-linear and the corresponding value for $x = 1{\cdot}48 \pm 0{\cdot}05$ within the range of enzyme and substrate concentrations investigated.

Enebo, Sandegren, and Ljungdahl (1953) using a barley cellulase and EHEC as substrate obtained the approximate relationship

$$U' \propto E^{2/3},$$

i.e. $x = 1{\cdot}5$. On the other hand, Preece and Aitken (1953) using a similar enzyme source, but with a barley β-glucosan substrate, found the ratio $E{:}U'$ to be constant,

TABLE 1

COMPARISON OF ENZYME CONCENTRATIONS AND ESTIMATED ACTIVITIES AT VARIOUS SCMC SUBSTRATE CONCENTRATIONS

Relative Enzyme Concentration	Enzyme Activity U' $\left(\text{i.e. } \frac{d(1/\eta_{sp})}{dt}\right) \times 10^2$ (min^{-1})		
	$(\eta_{sp})_{t=0} = 4{\cdot}33$	$(\eta_{sp})_{t=0} = 3{\cdot}60$	$(\eta_{sp})_{t=0} = 2{\cdot}60$
$16e$	119	164	241
$8e$	66	86	123
$4e$	38	50	62
$2e$	19	26	34
e	11	13	19

i.e. $x = 1$. These authors claimed that the apparent discrepancy between the two sets of results was a consequence of the use of higher enzyme concentrations by Enebo *et al.* However, in view of the results obtained with the assay of the *S. atra* cellulase against SCMC and EHEC, it appears that a more probable explanation lies in the use of different substrates.

A disadvantage of this method of assay is the difficulty in preparing solutions of reproducible substrate concentration and hence initial specific viscosity. Hultin's formula overcomes this obstacle by allowing for the variation in substrate concentration C. Since the viscosity of SCMC solutions is approximately proportional to the square of the concentration, a modification of Hultin's formula was examined, namely:

$$E = (\eta_{sp})^{y}_{t=0}(U')^{1{\cdot}1}. \qquad \ldots\ldots\ldots\ldots\ldots\ldots(4)$$

The value of the parameter y was estimated by analysis of the data in Table 1 to be $y = 1{\cdot}25 \pm 0{\cdot}07$. The results of the application of equation (4) to these data are shown in Table 3.

It can be seen that equation (4) accounts reasonably well for the influence on the value of U' of a variation in $(\eta_{sp})_{t=0}$, but it should be noted that this is only true when the variation in $(\eta_{sp})_{t=0}$ is due solely to a change in substrate concentration and not the degree of ploymerization (D.P.). Ingelman and Malmgren (1948) used a similar formula in which both x and $y = 1$ for the estimation of a polymetaphosphatase produced by *Aspergillus niger*.

TABLE 2

EVALUATION OF THE PARAMETER x RELATING ENZYME CONCENTRATION AND ACTIVITY AT VARIOUS SCMC SUBSTRATE CONCENTRATIONS

Initial Specific Viscosity ($(\eta_{sp})_{t=0}$)	$x\left(=\dfrac{\mathrm{d}\log E}{\mathrm{d}\log U'}\right)$
2·60	1·09
3·60	1·11
4·33	1·15

In their experiments on the assay of barley cellulase using a β-glucosan substrate, Preece and Aitken (1953) observed that for solutions of identical substrate concentration, variation in $(\eta_{sp})_{t=0}$, due presumably to a difference in D.P., did not affect the cellulase activity as determined by the slope U'. This follows from the linear relationship of $1/\eta_{sp}$ and t; thus $\mathrm{d}(1/\eta_{sp})/\mathrm{d}t$ (i.e. U') is a function of the rate of depolymerization and since it is constant, it should be independent of the initial D.P.

TABLE 3

COMPARISON OF ENZYME CONCENTRATIONS AND ESTIMATED ACTIVITIES AT VARIOUS SCMC SUBSTRATE CONCENTRATIONS

Relative Enzyme Concentration	Enzyme Activity $\left((\eta_{sp})^{1\cdot25}_{t=0}(U')^{1\cdot1}\right)\times 10$ (min^{-1})			Mean Activity
	$(\eta_{sp})_{t=0} = 4\cdot33$	$(\eta_{sp})_{t=0} = 3\cdot60$	$(\eta_{sp})_{t=0} = 2\cdot60$	
16e	77	88	88	84
8e	40	43	42	42
4e	22	23	20	22
2e	10·2	11·5	10·2	10·6
e	5·5	5·4	5·4	5·4

In the present investigation, the dependence of U' on substrate concentration and D.P. was tested using two grades of SCMC, namely high and medium viscosity grades. Solutions of equal concentration (0·5 per cent. w/v) were prepared, the differing viscosities of which were therefore a function of D.P. The high viscosity solution was designated A and the medium viscosity solution B. A third solution C of equal viscosity to B, was prepared by diluting A with the required proportion of water. Using a single dilute enzyme solution, U' was determined using these three substrate solutions (Table 4).

In agreement with the above considerations, solutions A and B yielded almost identical values for the slope U' despite their differing initial specific viscosities $(\eta_{sp})_{t=0}$. Furthermore, solution C, which had the same $(\eta_{sp})_{t=0}$ value as solution B gave a considerably higher slope U' due to the difference in substrate concentration. However, in accordance with equation (4), the values of $(\eta_{sp})_{t=0}^{1{\cdot}25}(U')^{1{\cdot}1}$ were similar for solutions A and C, since the difference in $(\eta_{sp})_{t=0}$ in this case was due solely to a concentration factor. Thus, although given a homogeneous batch of SCMC relative cellulase activities can be determined with reasonable accuracy, the values obtained for a single enzyme concentration using different batches of SCMC will vary with D.P., the degree of substitution, and the polydispersity of the substrate. In view of this complication, no attempt was made to define a unit of cellulase activity in terms of depolymerization rates.

TABLE 4

INFLUENCE OF CONCENTRATION AND DEGREE OF POLYMERIZATION OF SUBSTRATE ON THE DETERMINATION OF ENZYME ACTIVITY

Solutions A and B: 0·5%* w/v SCMC (high and medium viscosity grades respectively). Solution C: Solution A diluted to the same viscosity as solution B

Substrate Solution	Initial Specific Viscosity ($(\eta_{sp})_{t=0}$)	Enzyme Activity U' $\left(\text{i.e. } \frac{d(1/\eta_{sp})}{dt}\right) \times 10^2$ (min^{-1})	Enzyme Activity $\left((\eta_{sp})_{t=0}^{1{\cdot}25}(U')^{1.1}\right) \times 10$ (min^{-1})
A	1·53	32·5	4·9
B	0·80	32	2·1
C	0·73	78	5·1

* Low concentrations used to ensure complete solubility of SCMC.

The non-ionic cellulose derivative EHEC was compared with SCMC as a substrate for the viscometric determination of *S. atra* cellulase activity. A corresponding linearity between $1/\eta_{sp}$ and t was observed. However, in addition to its much lower sensitivity, it was found that the enzymic depolymerization characteristics of EHEC differed from those of SCMC. Thus if equation (4) is written in the form:

$$E = (\eta_{sp})_{t=0}^{y}(U')^{x}, \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots(5)$$

whereas for SCMC, $x = 1{\cdot}11 \pm 0{\cdot}01$ and $y = 1{\cdot}25 \pm 0{\cdot}07$, the corresponding values for EHEC (calculated from the data in Table 6) were $x = 1{\cdot}48 \pm 0{\cdot}05$ and $y = 1{\cdot}54 \pm 0{\cdot}09$. The results of assays using four serially diluted cellulase solutions (solutions 16*e*, 8*e*, 4*e*, and 2*e* of Table 2) and three EHEC solutions of differing concentrations, are set out in Table 5; calculations are based on

$$E = (\eta_{sp})_{t=0}^{1{\cdot}5}(U')^{1{\cdot}5}.$$

It can be seen, that with a knowledge of the values of the parameters x and y, equation (5) permits a reasonable comparison of cellulase activities even when assayed at different substrate concentrations. However, in view of the increased complexity in the calculation which this entails, for the purpose of the present investigation, wherever possible the activities were compared at a single substrate concentration.

(*b*) *Increase in Reducing Sugar*

The enzyme solution was incubated with a cellulosic substrate and the increase in soluble reducing sugar determined by the Nelson-Somogyi colorimetric method (Somogyi 1945). The substrates used were: (i) Whatman No. 1 filter paper, finely ground in a Wiley mill; (ii) acid-swollen cellulose, prepared by treating Whatman chromatographic cellulose powder (ashless grade) with phosphoric acid (85 per cent.) for 2 hr (cf. Walseth (1952*a*)). The final product was dialysed until free from yhosphate; (iii) SCMC or EHEC solutions.

Table 5

COMPARISON OF ENZYME CONCENTRATIONS AND ESTIMATED ACTIVITIES AT VARIOUS EHEC SUBSTRATE CONCENTRATIONS

Relative Enzyme Concentration	Enzyme Activities U' $\left(\text{i.e. } \frac{d(1/\eta_{sp})}{dt}\right)$ and E $\left(\text{i.e. } (\eta_{sp})_{t=0}^{1\cdot5}(U')^{1.5}\right)$					
	$(\eta_{sp})_{t=0} = 4\cdot48$		$(\eta_{sp})_{t=0} = 3\cdot09$		$(\eta_{sp})_{t=0} = 1\cdot83$	
	$U' \times 10^3$ (min^{-1})	$E \times 10^3$ (min^{-1})	$U' \times 10^3$ (min^{-1})	$E \times 10^3$ (min^{-1})	$U' \times 10^3$ (min^{-1})	$E \times 10^3$ (min^{-1})
16*e*	18	23	28	27	47	25
8*e*	13	15	19	14	32	14
4*e*	7·5	6·2	12	6·9	19	6·5
2*e*	4·4	3·2	6·8	3·1	13	3·6

(*c*) *Change in Birefringence of "Cellophane"*

The optical anisotropy of cellulose fibres is probably due to the alignment of cellulose chains within the fibre. In cellulose film (i.e. "Cellophane") the orientation of the chains is not completely random and consequently the film is birefringent. The change in birefringence which would be expected to occur during enzymic digestion of "Cellophane" has been investigated as a possible cellulase assay procedure.

A preliminary examination of the method was carried out using dilute sulphuric acid as the hydrolytic agent. No attempt was made to remove the "Cellophane" softener (usually glycerol or ethylene glycol). The retardation was measured using a polarizing microscope with a Berek compensator, as described in "McClung's Handbook of Microscopical Technique" (McClung Jones 1950). A 5-mm diameter "Cellophane" disc (300 gauge) was placed in a flat-bottomed glass dish and brought to focus in the centre of the field. The compensation angles (*a* and *b*) were determined and the difference ($2i$) used to calculate the retardation Γ and hence the birefringence B by means of the equation

$$\log \Gamma = \log Bl = \log C + \log f(i), \qquad \ldots\ldots\ldots\ldots (6)$$

where l = thickness of disc,
C = compensator plate constant, and
$\log f(i)$ = logarithmic function of i as supplied in the manufacturer's tables.

The disc was immersed in 50 per cent. sulphuric acid (v/v) at 26°C and the compensation angles measured continuously over a period of 20 min, by which time the disc had disintegrated. These results were used to calculate the decrease in retardation and the values obtained are listed in Table 6. No attempt was made to calculate the actual birefringence values, as this is dependent upon the thickness of the disc which undoubtedly changed during the degradation.

Table 6

INFLUENCE OF DILUTE SULPHURIC ACID ON THE RETARDATION OF BIREFRINGENT "CELLOPHANE"

Retardation measurements were carried out on a "Cellophane" disc immersed in 50% H_2SO_4 at 26°C

Reaction Time (min)	Difference $2i$ between Compensation Angles (degrees)	Retardation Γ (mμ)
0	19·0	215
2	14·6	127
4	9·9	58·5
6	7·4	32·7
8	5·8	20·0
10	4·6	12·6
12	3·6	7·7
14	2·9	5·0
16	2·4	3·4
18	2·1	2·6

On applying the method to the hydrolysis of "Cellophane" by *S. atra* culture filtrates, it was found that the decrease in birefringence was extremely slow. To obtain detectable changes, the procedure was modified so that the disc was gently shaken with the enzyme (at a rate which kept the disc in motion) for 20 hr at 37°C. It was then floated onto a microscope slide and placed under a cover slip. The difference in compensation angles was then measured.

A series of dilutions of a 15-fold concentrate of a *S. atra* culture filtrate was prepared using pH 5·0 McIlvaine citrate-phosphate buffer. These solutions were then assayed against "Cellophane"; the results are shown in Table 7. The results were reasonably reproducible, provided that the measurements were made in the centre of the disc and that the degradation was not allowed to proceed too far. When the value of the angle $2i$ decreased below 15°, the discs became very fragile and difficult to handle and the results of duplicate determinations varied appreciably. A sample of the enzyme concentrate which had been inactivated by autoclaving at 140°C for 30 min, did not alter the birefringence of the "Cellophane" during the usual 20 hr incubation period.

The procedure requires only semi-micro amounts of substrate and enzyme solution and as measurements of retardation do not involve any damage to the "Cellophane" disc, progressive changes occurring in a single sample can be followed. A further advantage arising from the use of "Cellophane" discs is the ease of measuring the surface area of the substrate; this factor is of importance in relation to heterogeneous kinetic studies.

TABLE 7

INFLUENCE OF SERIALLY DILUTED ENZYME SOLUTIONS ON THE RETARDATION OF BIREFRINGENT "CELLOPHANE"

"Cellophane" discs incubated with pH 5·0 buffered enzyme for 20 hr at 37°C

Enzyme Dilution	Difference between $2i$ Compensation Angles (degrees)	Retardation Γ (mμ)
1 in 2	16·1	155
1 in 4	17·0	164
1 in 8	17·6	184
1 in 16	18·9	213
1 in 32	20·1	240
1 in 64	20·2	242
1 in 128	20·1	240
1 in 256	20·5	250
∞	20·6	252

Unfortunately the technique is relatively insensitive, but it may well prove of value with concentrated cellulase solutions. With regard to mechanism studies, the method is of particular interest, since it provides a measure of one of the earliest stages of the digestion of crystalline cellulose, namely the disorientation of the cellulose chains. As the degree of orientation of the chains is largely determined by the forces exerted on the film during the manufacturing process, it should prove possible to prepare films covering a range of birefringence values.

IV. CONCENTRATION OF THE ENZYME

The culture medium was assayed daily against SCMC by the viscometric procedure. When the activity reached a peak value (*c.* 11 days, see Fig. 5), the solution was filtered, initially through butter muslin and then through Whatman No. 1 filter paper. The clear pale brown filtrate (20 l.) was concentrated in a climbing film vacuum evaporator at a temperature not exceeding 30°C, to a final volume of approximately 600 ml.

The concentrate was exhaustively electrodialysed between "Cellophane" membranes against a continuous flow of distilled water, until the conductivity of the solution was essentially constant (*c.* 10 hr). The solution was kept well mixed throughout the electrodialysis by means of a magnetic stirrer. The small residue which occasionally formed during this stage was separated by centrifuging; this residue was found to be devoid of cellulase activity. The supernatant solution when freeze-dried yielded an off-white powder (*c.* 2 g from 20 l. of culture filtrate), which contained at least 95 per cent. of the original activity as determined by the viscometric assay procedure.

V. General Properties of the Cellulase Concentrate

(*a*) *Heterogeneity*

(i) *Chemical Examination.*—The freeze-dried powder on analysis, proved to be free from ash and ammonium salts. A C, H, O, and N analysis* (O by direct assay) was carried out on a sample previously dried in a vacuum desiccator over P_2O_5. Found: C, 38·3; H, 6·7; O, 47·8; N, 7·2 per cent. (total 100·03 per cent.). Assuming that the nitrogen arose exclusively from protein, multiplication by the usual factor of 6·25 gave an approximate protein content of 45·2 per cent. On drying the analytical sample to constant weight at 80°C *in vacuo*, there was a further loss in weight of 6·9 per cent. which, if assumed to be due to the removal of water, increased the protein content of the dried powder to 48·6 per cent.

The presence of carbohydrate was indicated by a positive anthrone test, and analysis of the dried powder by the orcinol method of Pirie (1936) gave a value for the total carbohydrate content of 49·0 per cent. Thus the cellulase concentrate appeared to consist of approximately equal amounts of protein and carbohydrate.

Confirmation of the presence of protein was obtained by the detection of 14 amino acids in an acid hydrolysate prepared by digesting 50 mg of the freeze-dried powder in 6N HCl for 16 hr at 105°C. The hydrolysate was dried in a vacuum desiccator over KOH and P_2O_5 and the residue dissolved in approximately 0·1 ml of water. Using 5 μl of this solution, a two-dimensional paper chromatogram (Whatman No. 1 paper) was prepared using the solvents phenol-ammonia (22 hr) and butanol-acetic acid (18 hr). By means of a ninhydrin spray, the following 14 amino acids were detected: aspartic and glutamic acids, serine, glycine, histidine, lysine, arginine, threonine, alanine, proline, tyrosine, valine, phenylalanine, and leucine. The absence of cystine or methionine in the above series is particularly noteworthy.

The carbohydrate component was also examined by paper chromatography of an acid hydrolysate. The freeze-dried powder (54 mg) was gently refluxed with 0·33N H_2SO_4 (1 ml) for 2 hr, the hydrolysate then cooled, and the sulphate precipitated by the addition of a predetermined volume of 0·28N $Ba(OH)_2$. The $BaSO_4$ residue was removed by filtration and the pale yellow filtrate examined by paper chromatography on Whatman No. 1 paper, using the ethyl acetate-pyridine-water solvent of Jermyn and Isherwood (1949). Spraying with various reagents (ammoniacal silver nitrate, *p*-anisidine hydrochloride, naphthoresorcinol-trichloroacetic acid, and aniline hydrogen oxalate) clearly indicated the presence of appreciable quantities of galactose, mannose, and glucose (in that order of concentration) plus a trace amount of xylose. There was also present some slow moving material which was apparently ionic, since it was removed from the hydrolysate on passing through a "Bio-Deminrolit" mixed-bed, ion-exchange column. When sprayed with ninhydrin, this slow moving component developed a characteristic purple colour indicating the presence of a sugar-amino acid mixture or complex. No glucosamine could be detected using the above sprays and also the specific Morgan and Elson reagent (Partridge 1948). Amino sugars were also absent from the 6N HCl hydrolysate used to examine the amino acid composition of the preparation. Since much of the carbohydrate was destroyed as humin under conditions much more drastic than those outlined above, it is possible that amino sugars were present but not detected.

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* Analyses by Dr. W. Zimmerman, Microanalytical Laboratory, C.S.I.R.O., University of Melbourne.

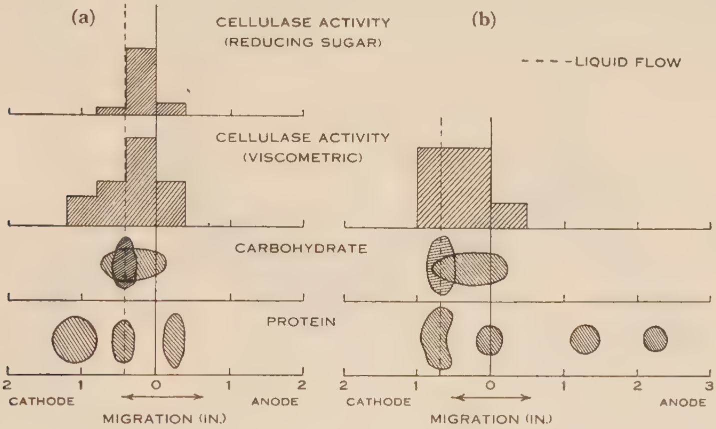

Fig. 7.—Paper electrophoresis of the enzyme concentrate (*a*) at pH 3·0 for 5 hr and (*b*) at pH 5·0 for 6 hr. Protein and carbohydrate components detected by spraying with 0·1 per cent. ninhydrin in ethanol and a saturated ethanolic solution of *p*-anisidine hydrochloride respectively, followed by heating at 105°C. Distribution of cellulase activity determined by eluting transverse strips (1 cm or 0·5 in. width) with pH 5·0 buffer and assaying the eluates viscometrically against SCMC. In an alternative assay procedure shown in (*a*), the increase in soluble reducing sugar on incubating each strip in pH 5·0 buffer for 16 hr was determined. ------ Liquid flow.

(ii) *Physical Examination.*—The techniques of paper electrophoresis and moving boundary electrophoresis were used to obtain further information on the heterogeneity of the cellulase concentrate.

The method of paper electrophoresis was that employed by Jermyn and Thomas (1954) in an examination of the multiple components of horse radish peroxidase. The runs were carried out in a cold room at 2°C, in order to reduce the operating temperature of the paper strip and consequently the extent of its interaction with the cellulolytic enzyme. The presence of protein was detected by drying the papers and developing with ninhydrin as for amino acids. The migration of the carbohydrate component was followed by spraying with *p*-anisidine hydrochloride in ethanol and heating at 105°C. To locate the cellulase activity, the paper strip was cut transversely into sections of 1 cm width, which were then placed in test tubes containing 5 ml of 0·1M sodium acetate buffer (pH 5) at 28°C and equilibrated by continuous shaking for 20 min. A 2-ml aliquot of each solution was assayed for activity against SCMC by the viscometric method. An alternative assay method was based on the direct interaction between the enzyme and the paper strips in acetate buffer. Thus the production of soluble reducing matter during an incubation period of 17 hr, provided a measure of the cellulase activity.

The results of paper electrophoresis at pH 3·0 (0·2N citric acid-sodium hydroxide) and pH 5·0 (0·1M sodium acetate-acetic acid) are illustrated diagrammatically in Figure 7. At pH 7·0 (0·1M sodium phosphate) and pH 8·5 (veronal buffer, ionic strength = 0·22) the patterns were similar to that obtained at pH 5·0. A run was attempted at pH 10, primarily with a view to studying the behaviour of the protein-carbohydrate complex which acts as though neutral at pH 3, 5, 7, and 8·5. Despite the poor staining qualities obtained at pH 10, distinct neutral protein and carbohydrate spots were visible, together with adsorbed immobile material. No carbohydrate trail or enzyme activity could be detected at this high pH.

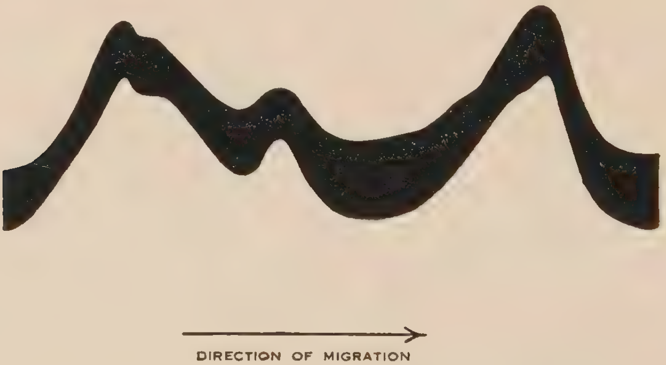

Fig. 8.—Moving boundary electrophoresis pattern: 0·9 per cent. freeze-dried electrodialysed enzyme in pH 8·54 veronal buffer, ionic strength = 0·22 at 1°C, 250 min, ascending boundary.

These results can be best interpreted on the assumption that the enzyme concentrate consists of at least three proteins and one or possibly two carbohydrates. A neutral, cellulase-active protein component appears to be complexed or at least closely associated with the non-adsorbed carbohydrate, neither showing any electrophoretic mobility, between pH 3 and 10. Of the remaining two protein components, one appears to have an isoelectric point below pH 3·0, while the isoelectric point of the other lies between pH 3·0 and 5·0; neither of these proteins could be shown to possess any cellulase activity on examination of the pH 5·0 paper electrophoresis strip. A possible second carbohydrate component appears at pH 5 and 7 as a trail extending from the origin to the neutral carbohydrate. This effect could be due to cellulase activity against the paper, either by the immobile enzyme adsorbed at the origin or else by the neutral protein-carbohydrate complex; however, this would not explain the apparent slight cathodic flow of this second carbohydrate (relative to the electro-osmotic flow) which was observed at pH 3.

Moving boundary electrophoresis at pH 8·54 confirmed the heterogeneity of the enzyme concentrate. The electrophoretic pattern is shown in Figure 8. The pattern indicated some immobile material plus at least three anodic components.

(*b*) *Substrate Specificity*

A cellulase preparation which rapidly depolymerized SCMC was tested for activity against various insoluble cellulosic substrates. These were (i) cotton duck, (ii) Whatman No. 1 filter paper, (iii) acid swollen cellulose, prepared as described above: (i) and (ii) were powdered in a Wiley mill and 1 per cent. aqueous suspensions of all three substrates prepared by agitating in a Waring Blendor. A 2-ml aliquot of each suspension was incubated with 5 ml of the enzyme solution and 5 ml of pH 5·0. McIlvaine buffer on a reciprocating shaker at 28°C. After 1 hr, the solutions were centrifuged and the supernatants assayed for reducing power. The results are given in Table 8.

Table 8

ENZYMIC HYDROLYSIS OF VARIOUS GLYCOSIDIC SUBSTRATES

Substrate and enzyme incubated in pH 5·0 McIlvaine buffer. Enzyme activity assayed as glucose* equivalent (in μg) of increase in reducing power of solution

Substrate	Incubation Time (hr)	Temp. (°C)	Enzyme Activity (Δ μg glucose*)
Cotton duck	1 and 60	28	0
Whatman No. 1 paper	1	28	57
Acid-swollen cellulose	1	28	70
Chitin	17	37	0
Xylan	17	37	50
Cellobiose	17	37	0

* In the cases of chitin and xylan, the reducing power was assayed as glucosamine and xylose equivalent respectively.

The inability of the enzyme to attack cotton duck was confirmed by prolonging the incubation period to 60 hr (2 drops of toluene were added to inhibit microbial growth), when no increase in soluble reducing matter could be detected. The cotton duck was a sample of material known to be susceptible to attack by *Memnoniella echinata* (Thomas 1955*b*). The case of hydrolysis of Whatman No. 1 paper and the even greater susceptibility of the acid-swollen cellulose, demonstrates the preferential digestion of amorphous cellulose by the *S. atra* enzyme.

The activity of the cellulase preparation towards cellobiose was also investigated as follows: enzyme concentrate (3 ml), pH 5·0 McIlvaine buffer (3 ml), and 0·01 per cent. cellobiose solution (3 ml) were incubated at 37°C for 17 hr. No increase in reducing power was observed during this period, indicating the absence of cellobiase activity. Xylanase and chitinase activities were also examined under the same conditions as in the cellobiase determination, the cellobiose solution being replaced by 100-mg samples of pear cell-wall xylan* and chitin. The results (Table 8) showed that the cellulase preparation possessed appreciable activity towards xylan but was inactive against chitin.

To determine the products of enzymic hydrolysis of cellulose, a mixture of an electrodialysed culture filtrate concentrate (20 ml) and a 3 per cent. dialysed suspension of phosphoric acid-swollen cellulose (20 ml), containing a few drops of toluene, was incubated at 37°C for 4 days. The pH of the mixture was 6·0 and the addition of buffer was deliberately avoided in order to eliminate the necessity for de-ionizing the solution prior to paper chromatographing the hydrolysis products. After 4 days, the solution was centrifuged and the supernatant assayed for reducing power; this was found to be equivalent to 0·71 mg/ml of glucose. The solution was concentrated to a minimum volume and chromatographed (6 hr at room temperature) as described above. Glucose and cellobiose were readily identified, a slower and much less concentrated spot was probably due to cellotriose, and a trace amount of an even slower component may well have corresponded to cellotetraose.

Table 9

CHROMATOGRAPHIC ASSAY OF THE PRODUCTS OF ENZYMIC HYDROLYSIS OF CELLULOSE

Enzyme and acid-swollen cellulose at pH 6·0 incubated for 4 days at 28°C and the hydrolysis products determined by paper chromatography

Hydrolysis Product	Glucose	Cellobiose	Cellotriose (?)	Cellotetraose (?)
Distance moved relative to glucose	1·00	0·62	0·40	0·23
Visual estimation of concentration	++++	+++++	+	±

These results (see Table 9) support the hypothesis of a cellulase capable of splitting both cellulose and water soluble oligoglucosides in a largely random manner. To test the ability of the enzyme to hydrolyse the lower oligoglucosides, it was assayed against cellotriose and cellotetraose.† A concentrated solution of the freeze-dried enzyme (5 mg/ml) and 0·625 mM solutions of the substrates cellobiose, cellotriose, and cellotetraose were prepared in pH 5·0 acetate buffer. After adjusting the temperature of the solutions to 38·9°C, 1 ml of enzyme was added to 4 ml of each substrate; 1-ml samples of the mixtures were removed at intervals during the 2-hr incubation period and added to an equal volume of Nelson-Somogyi reagent to stop the reaction. The reducing sugar contents of these samples were determined in the usual manner and the results are shown in Table 10.

Thus although the *S. atra* cellulase is inactive against cellobiose, both cellotriose and cellotetraose are readily split and, as in the case of the cellulase of *Myrothecium*

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* The xylan was purified by Dr. M. A. Jermyn, to whom the author is indebted for a sample.

† The author is grateful to Dr. D. R. Whitaker of the National Research Council, Ottawa, for samples of pure cellotriose and cellotetraose.

verrucaria (Whitaker 1954) the tetrasaccharide is split more rapidly than the trisaccharide.

(*c*) *Influence of pH*

A sample of culture filtrate, containing a small amount of toluene, when stored at pH 6·5 and a temperature not exceeding 10°C, showed no loss in activity over a period of 2 months. However, the dialysed solution (pH 6·1) was less stable, and when stored under the same conditions for 2 weeks an appreciable decrease in activity resulted.

The influence of pH on the cellulase activity of an electrodialysed enzyme preparation was investigated under differing sets of conditions: (i) using Whatman No. 1 filter paper, previously ground to a powder in a Wiley mill. The activities were determined by the increase in soluble reducing matter; (ii) using solutions of EHEC as substrate. The activities were determined both by the increase in soluble reducing matter and by the viscometric assay procedure. The results are shown in Figure 9. In an investigation of the stability of the cellulase at various pH values, a second assay series was carried out using enzyme solutions mixed with equal volumes of buffers and incubated at 37°C for 16 hr prior to assaying (toluene was added to each solution to prevent microbial growth). The corresponding set of curves are indicated by full circles in Figure 9.

Table 10

ENZYMIC HYDROLYSIS OF CELLOBIOSE, CELLOTRIOSE, AND CELLOTETRAOSE

Enzyme incubated with substrate in pH 5·0 buffer at 38·9°C; the extent of hydrolysis was followed by the increase in reducing power*

Incubation Time (min)	Percentage Increase in Initial Reducing Power*		
	Cellobiose	Cellotriose	Cellotetraose
1 (approx.)	2	0	16
5	—	—	60
20	—	32	74
60	1	53	76
120	5	61	—

* Calculated as increase in optical density of the solution after treatment with Nelson-Somogyi reagent.

It can be seen from the first assay series, that the influence of pH on the activity of the enzyme towards both substrates is similar, when assayed by the reducing sugar method. Thus, under the above experimental conditions, the maximum activity at 37°C was obtained between pH 6 and 7·5. In the case of EHEC the viscometric assay procedure yielded a somewhat different pH-activity curve with a higher pH optimum, although these tests were carried out at a lower temperature (30°C) and with a considerably shorter incubation period.

Comparison with the second (pre-incubated) assay series illustrates the relative stability of the cellulase over an appreciable pH range. Thus the discrepancy between the pH-activity curves obtained by the viscometric and reducing sugar assay methods cannot be accredited to the considerable difference in incubation times.

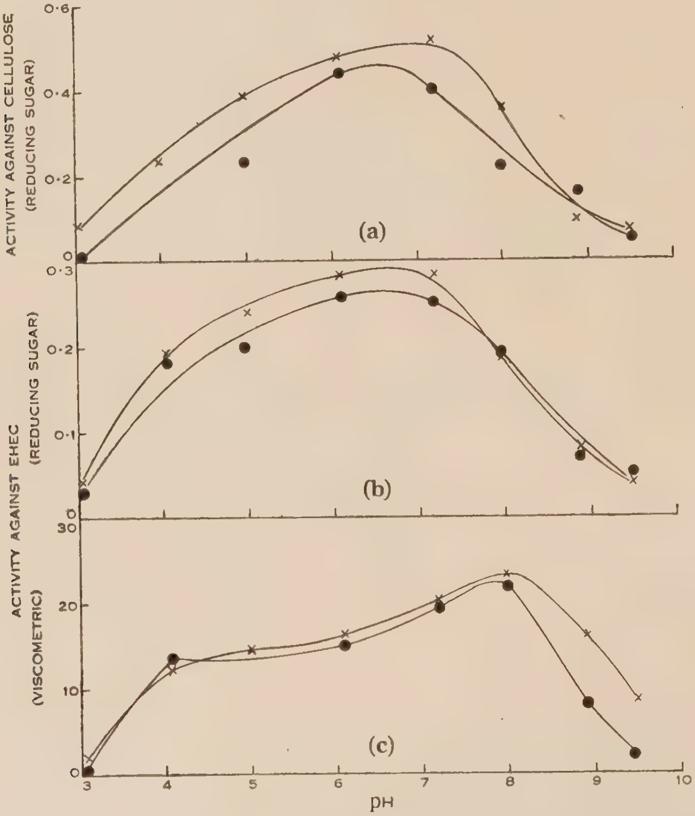

Fig. 9.—Influence of pH on cellulase activity. (*a*) Increase in soluble reducing sugar (arbitrary units) on incubating powdered Whatman No. 1 paper (100 mg) with pH 5·0 buffered enzyme (4 ml) for 16 hr at 37°C. (*b*) Activity against EHEC; conditions as in (*a*), but with 1·5 per cent. w/v EHEC solution (2 ml) as cellulosic substrate. (*c*) Activity against EHEC: assayed viscometrically in pH 5·0 buffer at 30°C (N.B.—Total assay time less than 15 min.) Buffers used: pH 3-8, McIlvaine; pH 8-10, borate. × Assay using freshly prepared enzyme solution. ● Assay using enzyme solution previously maintained at 37°C for 16 hr.

(*d*) *Inhibition of Cellulase Activity*

The influence of low concentrations of various sugars and other compounds on cellulase activity was investigated using the SCMC viscometric assay technique. In these experiments, a dialysed cellulase concentrate was diluted with 20 volumes of a solution of the test compound and then assayed in the normal manner. The results (Table 11) show that of the compounds tested, mercuric acetate alone completely inhibited the activity, although partial inhibition was produced by copper sulphate, lead acetate, and potassium cyanide. None of the sugars influenced the activity at the above concentrations.

(*e*) *Adsorption on Cellulose*

In order to determine the degree of adsorption of the cellulase of *S. atra* by cellulose, an electrodialysed solution of the enzyme (5 ml) was mixed with solutions of pH 5·0 McIlvaine buffer (7 ml) containing: (i) 1·0 g Whatman chromatographic powder; (ii) 0·1 g phosphoric acid-swollen cellulose. After equilibrating at 37°C for 30 min each solution was centrifuged and the activity of the supernatant assayed viscometrically against SCMC and compared with that of a control solution which did not contain any cellulose.

Table 11

THE INHIBITION OF CELLULASE ACTIVITY BY VARIOUS SUBSTANCES

Enzyme activity assayed viscometrically against SCMC in pH 5·0 buffer at 30°C

Reagent	Concentration (%)	Percentage of Original Activity
Glucose	0·2	100
Galactose	0·2	100
Mannose	0·2	100
Xylose	0·2	100
Arabinose	0·2	100
Cellobiose	0·2	100
Maltose	0·2	100
Lactose	0·2	100
Mercuric acetate	0·02	0
Cupric sulphate	0·02	60
Chromic sulphate	0·02	100
Lead acetate	0·02	66
Potassium cyanide	0·02	70
Hydroxylamine	0·02	100
8-Hydroxyquinoline	0·02	100
Phenylhydrazine	0·02	100
Thiosemicarbazide	0·02	100
Girard's P reagent (pyridinium acethydrazide chloride)	0·02	100
Iodoacetic acid	0·02	100

Of the original activity, 87 per cent. was retained in the control, but only 30 and 43 per cent. was retained after incubation with the Whatman chromatographic powder and the acid-swollen cellulose respectively. The amount of soluble reducing matter formed in 30 min at 37°C was relatively small, i.e. in (i) 58 μg/ml, and in (ii) 150 μg/ml calculated as glucose. The inhibition experiments in the previous section show that these small amounts are unlikely to have significantly affected the activity measurements. It was not confirmed that a reversible adsorption occurred and it is possible that at least part of the loss in activity was due to denaturation at the cellulose surface. However, this is improbable in view of the fact that one of the cellulose samples tested was a constituent of the mould culture medium.

(*f*) *Precipitability*

The effects of adding ammonium sulphate, ethanol, and polymethacrylic acid to culture filtrate concentrates were investigated with a view to devising a cellulase purification procedure.

Ammonium sulphate precipitation experiments were carried out on a 10-fold concentrate of the culture filtrate (pH 6·0). Varying amounts of saturated ammonium sulphate at 8°C were added to the enzyme concentrate at the same temperature, the final volumes of the mixtures being 10 ml. After 60 min the precipitates were separated by centrifuging and then redissolved in 10 times the original enzyme volume of pH 5·0 McIlvaine buffer. The cellulase activity of each solution was assayed viscometrically and compared with that of a control consisting of the original enzyme concentrate diluted 10-fold in buffer. The results were reproducible and a typical curve is shown in Figure 10.

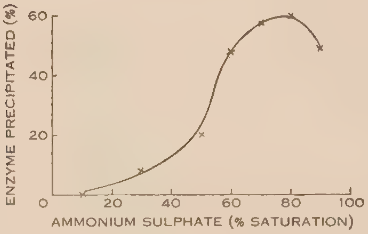

Fig. 10

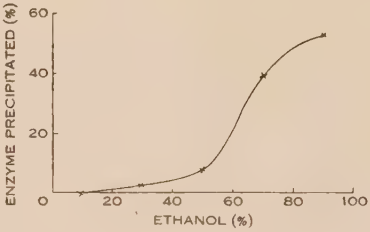

Fig. 11

Fig. 10.—Ammonium sulphate precipitation of cellulase from culture filtrate concentrate. Residues dissolved in pH 5·0 buffer and assayed viscometrically against SCMC at 30°C.

Fig. 11.—Ethanol precipitation of cellulase from culture filtrate concentrate. Residues dissolved in pH 5·0 buffer and assayed viscometrically against SCMC at 30°C.

A negligible precipitate was obtained until 40 per cent. saturation was reached; at 80 per cent. saturation approximately half of the total activity was precipitated. Addition of further ammonium sulphate decreased the yield of enzyme, possibly owing to denaturation. A paper-electrophoretic examination of a solution of the material precipitated at 80 per cent. saturation showed that it contained all the components of the initial culture filtrate concentrate, including the carbohydrate.

In a parallel series of tests, addition of ethanol to the undialysed concentrate at —5 C° and pH 6·0 precipitated no more than a part of the enzyme over a period of 2 hr (see Fig. 11). The precipitability of the enzyme with ethanol at low ionic strength was investigated using a 10-hr electrodialysate of the above culture filtrate concentrate. No precipitate separated from the solution (pH 5·0) even at an ethanol concentration of 80 per cent. (v/v) when left to stand overnight at —5°C. On readjusting the salt concentration to approximately its original value by the addition of a Waksman-Carey culture medium concentrate, the ability of ethanol to partially precipitate the enzyme was restored to the original level shown in Figure 11.

Polymethacrylic acid has been used by Whitaker (1953) to precipitate the cellulase of *Myrothecium verrucaria.* A precipitation of the cellulase of *S. atra* was attempted under closely parallel conditions, using a sample of polymethacrylic acid (PMA) generously supplied by Dr. Whitaker. A sample of freeze-dried electrodialysed enzyme (50 mg) was dissolved in 20 ml of sodium acetate-acetic acid buffer, pH 4·35, of ionic strength 0·01 at 5°C; a 0·25 per cent. PMA solution was prepared using the same buffer. Up to two volumes of the PMA solution were carefully added to the enzyme, but no precipitate formed even on standing overnight at 5°C.

VI. Discussion

The cellulolytic activity of *S. atra* is due to a relatively stable extracellular enzyme which is capable of hydrolysing a considerable range of β-1, 4′-polyglucosides. This cellulase is produced in appreciable concentration when the mould is grown on cellulosic substrates, but on a glucose medium it appears only in trace amounts. Thus in this case it behaves as an adaptive enzyme.

The initial enzyme concentrate contains at least three electrophoretically distinguishable proteins in addition to one or perhaps two carbohydrate components. Two of the proteins do not appear to be active against filter-paper cellulose or SCMC, and the remaining active component is intimately associated with the carbohydrate moiety. The major carbohydrate component seems to mask the charge on the protein, since on paper electrophoresis at various pH values these two components moved together as a single neutral spot. Thus although paper electrophoresis provides some evidence of a single cellulase component, the neutral carbohydrate may possibly be associated with more than one protein. Jermyn (1955) has found that the *p*-nitrophenyl-β-glucosidase produced by *S. atra* when grown on soluble carbon sources, is associated with a similar, if not identical, carbohydrate. The carbohydrate may well be a normal metabolic product of the mould, which is not directly related to the enzyme activity. Jermyn found the carbohydrate to be essential to the stability of the β-glucosidase but not to its activity.

Attempts to separate the enzyme by precipitation with ammonium sulphate, ethanol, or PMA were unsuccessful. The relatively high solubility of the crude enzyme in solutions of these reagents, could also be due to the influence of the carbohydrate on the properties of the protein components.

The carbohydrate on acid hydrolysis yielded galactose, mannose, glucose, and a small proportion of xylose. This is of interest, since it illustrates the ability of *S. atra* to convert the glucose residues of cellulose into other sugars. A related fungal polysaccharide, capreolinose, has been previously isolated from *Penicillium capreolinum*, the constituent residues being mannose, glucose, galactose, and malonic acid (Clutterbuck 1936).

A noteworthy feature of a qualitative amino acid analysis of the crude enzyme was the absence of the sulphur-containing acids cysteine (or cystine) and methionine.

This observation is in keeping with the failure of the thiol-specific reagent, iodoacetic acid, to inhibit the cellulase activity. In this respect, the cellulase of *S. atra* apparently differs from that of *Myrothecium verrucaria*, which was shown by Basu and Whitaker (1953) to be inhibited by iodoacetic acid and other thiol-specific inhibitors. The failure of phenylhydrazine to inhibit the viscometric activity is also of interest, in view of the high fungal resistance of phenyhydrazine-treated cotton duck in which this reagent is coupled at very low degrees of substitution (Thomas 1955*b*).

The *S. atra* cellulase is active over the pH range 4-9, the optimum pH varying between 6·5 and 8, depending upon the substrate and assay method. As with many other cellulolytic enzymes (Norkrans 1950; Walseth 1952*b*), the activity of the *S. atra* cellulase is inversely proportional to the crystallinity of the cellulose substrate. It can hydrolyse both soluble and insoluble β-1,4′-polyglucosides ranging from cellotriose and the soluble cellulose derivatives SCMC and EHEC to phosphoric acid-swollen cellulose and filter paper. However, no activity could be detected against cellobiose or the highly crystalline cellulose of cotton duck. Xylan (a β-1,4′-polyxyloside) is also hydrolysed, but in view of its close structural relationship to cellulose, this may well be an indication of the group specificity of the enzyme, rather than evidence of an independent xylanase.

The products of an extended enzymic hydrolysis of cellulose consisted of a mixture of cellobiose and glucose, with very small amounts of two other sugars, possibly cellotriose and cellotetraose. This result supports the hypothesis of a single enzyme capable of hydrolysing cellulose chains in a random manner, since in the absence of cellobiase activity, a hydrolysis of this type would ultimately yield equimolecular amounts of glucose and cellobiose. A random hydrolysis mechanism would also explain the ease of depolymerization of soluble cellulose derivatives by dilute solutions of this enzyme. Qualitative examination of paper chromatograms of the hydrolysis products indicated an excess of cellobiose. This could be explained by the presence of a second enzyme of the β-amylase type, capable of removing cellobiose units end-wise from the cellulose chains. However, there is an alternative possibility which does not necessitate the presence of a second enzyme. Thus, if the specificity of the enzyme is such that the rate of hydrolysis of terminal glucosidic bonds is less than the rate for internal bonds, splitting of the lower oligoglucosides would favour the formation of cellobiose at the expense of glucose. Recently (Whitaker 1954) in a detailed examination of the enzymic hydrolysis of a series of β-1,4′-oligoglucosides by a purified cellulase extract of *Myrothecium verrucaria*, obtained strong evidence in favour of a terminal bond specificity. His data particularly favoured the hypothesis that one terminal linkage is split less frequently than the other. If this is true in the case of the *S. atra* cellulase, it would help to explain the observation that, although this enzyme is apparently incapable of degrading cellobiose, it will readily hydrolyse cellotriose and cellotetraose.

A notable difference between the cellulases of these two moulds, in addition to that mentioned earlier, is the lack of any evidence of cellobiase activity in the case of *S. atra*. However, in view of the heterogeneity of the *S. atra* enzyme preparation, detailed comparisons with cellulases from other sources would be premature at this stage.

VII. ACKNOWLEDGMENTS

The author is indebted to Mr. A. B. McQuade for collaboration in the development and production of bulk cultures of *S. atra*; to Mr. G. C. Hawkes for valuable technical assistance; to Dr. R. D. B. Fraser for advice in relation to birefringence studies; to Mr. J. M. Gillespie for carrying out the moving boundary electrophoresis determination; and to Mr. B. Hall for detailed statistical examination of the viscometric assay data.

VIII. REFERENCES

BASU, S. N., and WHITAKER, D. R. (1953).—*Arch. Biochem. Biophys.* **42**: 12.
CLUTTERBUCK, P. W. (1936).—*J. Soc. Chem. Ind.* **55**: 55T.
ENEBO, L., SANDEGREN, E., and LJUNGDAHL, L. (1953).—*J. Inst. Brew.* **59**: 205.
HULTIN, E. (1946).—*Svensk. Kem. Tidskr.* **58**: 198.
HULTIN, E. (1949).—*Acta Chem. Scand.* **3**: 625.
INGELMAN, B., and MALMGREN, H. (1948).—*Acta Chem. Scand.* **2**: 365.
JERMYN, M. A. (1953).—*Aust. J. Biol. Sci.* **6**: 48.
JERMYN, M. A. (1955).—*Aust. J. Biol. Sci.* **8**: 541.
JERMYN, M. A., and ISHERWOOD, F. A. (1949).—*Biochem. J.* **44**: 402.
JERMYN, M. A., and THOMAS, R. (1954).—*Biochem. J.* **56**: 531.
LEVINSON, H. S., and REESE, E. T. (1950).—*J. Gen. Physiol.* **33**: 601.
MCCLUNG JONES, R. (1950).—"McClung's Handbook of Microscopical Technique." 3rd Ed. p. 648. (Paul B. Hoeber Inc.: New York.)
NORKRANS, B. (1950).—*Physiol. Plant.* **3**: 75.
PARTRIDGE, S. M. (1948).—*Biochem. J.* **42**: 240.
PIRIE, N. W. (1936).—*Brit. J. Exp. Path.* **17**: 269.
PREECE, I. A., and AITKEN, R. A. (1953).—*J. Inst. Brew.* **59**: 453.
SANDEGREN, E., and ENEBO, L. (1952).—*J. Inst. Brew.* **58**: 198.
SOMOGYI, M. (1945).—*J. Biol. Chem.* **161**: 89.
THOMAS, R. (1955*a*).—*Aust. J. Chem.* **8**: 142.
THOMAS, R. (1955*b*).—*Text. Res. J.* **25**: 559.
TRACEY, M. V. (1950).—*Biochem. J.* **47**: 431.
WALSETH, C. S. (1952*a*).—*T.A.P.P.I.* **35**: 228.
WALSETH, C. S. (1952*b*).—*T.A.P.P.I.* **35**: 233.
WHITAKER, D. R. (1953).—*Arch. Biochem. Biophys.* **43**: 253.
WHITAKER, D. R. (1954).—*Arch. Biochem. Biophys.* **53**: 439.

NON-UTILIZATION OF ACETATE AND UTILIZATION OF GLUCOSE BY THE BRAIN OF THE SHEEP

By G. L. McClymont* and B. P. Setchell†

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Summary

Arteriovenous difference studies on the brain of the sheep have failed to show any utilization of acetate. The mean glucose uptake by the brain, 6·2±0·7 mg per 100 ml of blood, was sufficient, assuming complete oxidation of the glucose, to account for the mean oxygen utilization of 4·8±0·2 vol. per 100 ml of blood.

I. Introduction

The quantitative importance of acetate as a peripheral metabolite of ruminants (Reid 1950; McClymont 1951*a*) has been established. *In vitro* studies with non-ruminants have shown that acetate is not oxidized by the brain (Elliott, Greig, and Benoy 1937; McGowan and Peters 1937; Elliott, Libet, and McNair Scott 1942) and may in fact be produced by brain tissues when pyruvate is used as the substrate (Long 1938; Elliott *et al*. 1942).

With the tentative identification of ovine pregnancy toxaemia as a hypoglycaemic encephalopathy (McClymont and Setchell 1955), it became of particular interest to determine whether the brain of the sheep is similar to that of non-ruminants, in which glucose is the only major catabolite (Himwich 1951), or whether the metabolic adaptations to ruminal digestion have proceeded to the extent of allowing acetate utilization by the brain.

Arteriovenous difference studies now reported show that the sheep is similar to non-ruminants in that acetate is not utilized by the brain, and that the mean glucose uptake, assuming complete oxidation of the glucose is sufficient to account for the mean oxygen uptake.

II. Experimental

Sheep studied were normal Corriedale ewes which had been fed *ad lib.* until immediately before the experiment, or only fasted overnight.

Arterial blood was taken from the femoral or carotid artery and venous blood from the dorsal sagittal sinus. The sinus was exposed by trephining the skull in the mid line about 5 cm from the occipital crest. Samples were either taken under light nembutal anaesthesia, following exposure of the sinus and the carotid or femoral artery, or from unanaesthetized animals with carotid loops and permanent polythene cannulae inserted in the sinus. There was no detectable difference in results from the two techniques.

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Blood samples were taken simultaneously and at the same rate from the artery and sinus, taking up to half a minute to take each set of samples. Sodium citrate, 0·5 ml of a 4 per cent. solution, was used to prevent clotting in the syringes, and a drop of mercury in the syringe used for mixing the blood. Samples for oxygen determination were immediately injected under paraffin, and samples for glucose determination immediately injected into bottles containing solid sodium fluoride. Volatile fatty acid in the blood, expressed as acetic acid in view of the predominance of this acid in the volatile fatty acid fraction in ruminant blood (Reid 1950; McClymont 1951*b*), was determined by the method of Scarisbrick (1952). Oxygen was determined by the van Slyke manometric technique (Hawk and Bergeim 1937), and glucose by the method of Somogyi (1945, 1952). All analyses were done in duplicate or triplicate.

TABLE 1

ACETATE* ARTERIOVENOUS DIFFERENCES IN THE SHEEP'S BRAIN

Sheep No.	Artery (mg %)	Sagittal Sinus (mg %)	Arteriovenous Difference (mg %)
1	4·2	4·4	−0·2
	4·5	4·7	−0·2
	4·4	4·8	−0·4
	2·3	2·3	0
	1·8	1·8	0
2	4·1	4·0	0·1
	4·1	4·3	−0·2
	4·5	4·2	0·3
3	7·1	6·9	0·2
	7·6	7·2	0·4
4	6·4	7·1	−0·7
Mean	4·64	4·70	−0·06
4	6·4	3·1†	3·3

* Expressed as acetic acid.

† Taken from femoral vein simultaneously with arterial and sinus samples of sheep No. 4.

III. RESULTS

The results of 11 acetate and 25 glucose arteriovenous difference estimations are shown in Tables 1 and 2. No appreciable uptake of acetate by the brain could be demonstrated, the small positive and negative differences in individual determinations being within the error of the method. The data of Reid (1950) indicate that at an arterial acetate level of 4·6 mg per cent., the mean of the values in the present work, the mean jugular acetate arteriovenous difference would have been *c.* 1·2 mg per cent. The large arteriovenous difference observed on the femoral vein is shown for comparison, this difference being in conformity with the observations of Reid (1950) and McClymont (1951*a*).

Glucose uptake was considerable, mean 6·2±0·7 mg per cent., but there was considerable variation in the magnitude of the differences, even between samples from the one sheep taken within several minutes of one another, and despite relatively constant oxygen uptake, as the following figures show:

Time	Arteriovenous Difference of Glucose (mg %)	Arteriovenous Difference of Oxygen (vol. %)
11·0	8·2	5·4
11·3	5·7	5·5
11·7	5·3	5·5
11·10	10·5	5·7

The coefficient of variation of glucose uptake was 0·61. Oxygen utilization was much more constant, the mean being 4·9±0·2 vol. per cent., and the coefficient of variation only 0·17.

TABLE 2

GLUCOSE AND OXYGEN ARTERIOVENOUS DIFFERENCES IN THE SHEEP'S BRAIN, BASED ON 25 OBSERVATIONS ON EIGHT SHEEP

	Glucose			Oxygen			Glucose-Oxygen Ratio for Each Arteriovenous Difference Determination
	Artery (mg %)	Sagittal Sinus (mg %)	Arteriovenous Difference (mg %)	Artery (vol. %)	Sagittal Sinus (vol. %)	Arteriovenous Difference (vol. %)	
Mean	54·7±2·3	48·5±2·4	6·2±0·7	8·9±0·36	4·1±0·30	4·8±0·20	1·28±0·18
Range	31·6-72·5	28·0-64·3	−1·3-16·2	4·7-12·3	1·3-6·5	3·1-5·8	0·42-3·82

It seems likely that the greater variability in glucose uptake was related to short term changes in blood glucose levels which resulted in different relative intra- and extravascular glucose concentrations, the differences probably tending to be higher with any increase in blood glucose levels and lower with any decrease, due to resulting changes in diffusion potentials. Short term fluctuations in brain glycogen concentrations may also have been involved.

The theoretical ratio of glucose (mg per cent.) to oxygen (vol. per cent.), for complete oxidation of glucose, is 1·34. The mean value of the ratios for the 25 observations was 1·28±0·18, the high standard error being largely caused by the wide fluctuations in glucose uptake. Whilst the standard error of this ratio makes impossible any exact comparison with the theoretical ratio, the data nevertheless strongly suggest that the glucose uptake by the brain of the sheep was sufficient to account for the oxygen utilization by the brain.

IV. Discussion

Despite the metabolic adjustments of the sheep apparently directed at minimizing glucose utilization, such as a low blood glucose, reduced insulin sensitivity (Reid 1951), and low glucose arteriovenous differences (Reid 1950), the evolution of the sheep has evidently not proceeded to the extent of the qualitative adaptation involved in permitting acetate utilization by the brain. While the variability of the glucose uptake rather vitiated quantitative equating of glucose and oxygen uptakes, nevertheless the data suggest that the brain of the sheep is similar to that of other animals, with glucose as the sole major catabolite. The arteriovenous differences recorded in these sheep were somewhat lower than in man, values for glucose and oxygen differences recorded by Gibbs *et al.* (1942) being 9·8 mg per cent. and 6·7 vol. per cent., and considerably lower than for the dog, 13·1 mg per cent. and 9·3 vol. per cent. (Himwich and Fazekas 1937).

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VI. References

Elliott, K. A. C., Greig, M. E., and Benoy, R. P. (1937).—*Biochem. J.* **31**: 1003.

Elliott, K. A. C., Libet, B., and McNair Scott, D. B. (1942).—*J. Biol. Chem.* **146**: 251.

Gibbs, E. S., Lennox, W. G., Nims, L., and Gibbs, F. A. (1942).—*J. Biol. Chem.* **144**: 328.

Hawk, P. B., and Bergeim, D. (1937).—"Practical Physiological Chemistry." p. 533. (The Blakiston Co.: Philadelphia.)

Himwich, H. E. (1951).—"Brain Metabolism and Cerebral Disorders." (The Williams & Wilkin Co.: Baltimore.)

Himwich, H. E., and Fazekas, J. F. (1937).—*Endocrinology* **21**: 800.

Long, C. (1938).—*Biochem. J.* **32**: 1711.

McClymont, G. L. (1951*a*).—*Aust. J. Agric. Res.* **2**: 158.

McClymont, G. L. (1951*b*).—*Aust. J. Agric. Res.* **2**: 92.

McClymont, G. L., and Setchell, B. P. (1955).—*Aust. Vet. J.* **31**: 53.

McGowan, C. K., and Peters, R. A. (1937).—*Biochem. J.* **31**: 1637.

Reid, R. L. (1950).—*Aust. J. Agric. Res.* **1**: 338.

Reid, R. L. (1951).—*Aust. J. Agric. Res.* **2**: 132.

Scarisbrick, R. (1952).—*Biochem. J.* **50**: xxxiv.

Somogyi, M. (1945).—*J. Biol. Chem.* **160**: 61.

Somogyi, M. (1952).—*J. Biol. Chem.* **195**: 19.

INHERITANCE OF ANTIBODY RESPONSE

II. MEASUREMENT OF RESPONSE

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Summary

The haemolytic titre as estimated by a dilution series is a very precise measure of response to antigenic stimulation when compared with biological variation.

Such variables as age of complement, level of complement, age and source of red cells, storage prior to reading, dose of red cells, and time of obtaining antisera are additive in their effect on titre. Valid comparisons may be made under an arbitrarily selected combination of these.

Both primary and secondary time-response curves have a biphasic form which is further discussed below.

I. Introduction

A beginning has been made in studying the quantitative inheritance of the ability to produce antibodies (Sang and Sobey 1954; Sobey 1954). If the underlying genetic parameters are to be elucidated (see, for example, Mather 1949), the animals used must be kept under some degree of laboratory control and measurements of responses of many individuals made. For this reason very refined but time consuming laboratory determinations of response to antigenic stimulation are precluded and rapid methods of estimation must be used. This is of little consequence if errors of measurement are small compared with the variation between animals.

The modern method of experimentation introduced by Fisher about 30 years ago (Fisher 1955) is well suited to the problem of determining an optimal technique of measurement. It enables, amongst other things, the rapid elimination of factors irrelevant to the method. This approach has never before been employed in the development of immunological methods and it is shown that it leads to concise experimentation.

In this paper a series of experiments has been analysed to show to what extent the responses of mice and rabbits to injection of sheep red cells are affected by factors other than differences between individuals; and whether such effects are an important source of error when differences between individuals are estimated. The response of the animals is measured by the extent to which their immune sera, in the presence of guinea pig complement, will haemolyse sheep red cells. Factors which have been investigated, other than individual differences between animals are age, source and dose of red cells, age and level of complement, storage of tubes prior to reading, and the time interval between injection and collection of antiserum. Mice were used

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where large numbers were required for analysis, and rabbits where large volumes of serum were necessary.

II. Experimental Methods and Results

Methods of preparing red cells for injection, collection of immune serum and complement, and the routine of testing immune serum have been described in a previous paper (Sobey and Adams 1955*a*).

Table 1

SUMMARY OF THE DOSE-RESPONSE AND TIME-RESPONSE DATA FOR BOTH PRIMARY AND SECONDARY RESPONSES

Each entry in the table is the mean of five observations

Response	Dose (%)	Time (days)				
		3	6	9	12	15
Primary	0·1	8·1	12·0	7·9	8·4	7·5
	0·01	4·1	9·1	5·4	6·9	5·9
	0·001	0·1	7·5	5·0	6·2	2·2
	0·0001	0·1	1·8	1·9	2·0	0·1
Secondary	0·1	11·0	14·4	11·5	9·9	10·1
	0·01	11·1	12·9	10·1	10·1	8·7
	0·001	7·6	13·6	11·0	6·6	9·3
	0·0001	6·2	7·0	9·3	6·6	8·0

(*a*) *Dose-response and Time-response Curves*

A total of 100 female albino mice about 28 weeks old taken from a random-bred population were used in this experiment. The mice were randomly allotted to 20 boxes, five per box. Five boxes of mice were allotted to each of four dose groups so that 25 mice received each dose level. Doses of 0·5 ml, of 0·1, 0·01, 0·001, and 0·0001 per cent. packed sheep red cells in normal saline were injected into the tail vein. Injections were made with fresh sheep red cells obtained from a single sheep. Mice were bled 3, 6, 9, 12, or 15 days after injection. All mice were bled on the same day over a period of 3 hr. Five mice from each dose level were allotted to each time interval, each mouse being bled only once. Time-response curves were not determined with individual mice, in order to preserve statistical independence of the dose and time components of response.

Since no natural haemolysins of sheep red cells have been found in mice (Sobey and Adams 1955*a*) it may be presumed that we are dealing with a primary response. A summary of the results and the analysis of variance are given in Tables 1 and 2 respectively. The lowest dosage group was omitted from the analysis since many animals failed to respond. The analysis indicates that there is linear dependence of log titre (hereinafter referred to as "titre") on log dose. The slope of the dose-response line of the primary response depends on the time of bleeding; the differences in slope at the five different time intervals are shown to be significant by the linear ×times interaction. The time-response curves contain two peaks. The results are illustrated in Figure 1, the response surface intersecting the zero response ordinate if the lowest dose, which has been omitted, is included. The surface shown is based on the regression equation obtained using the significant effects of the analysis.

After a period of 1 week the animals were again randomly allotted to 20 boxes and the same experimental plan repeated. This ensures that the secondary responses observed were not biased by the levels of primary response. The experimental results and the analysis of variance are given in Tables 1 and 2. It will be noted in Table 1 that any secondary response mean is always greater than the corresponding primary response mean. At all times of bleeding there is a strong linear relationship between titre and log dose. The slope of these dose-response lines depends on time, there being more violent slope fluctuations than with the primary responses. Again there is a complex biphasic time-response curve which is illustrated in Figure 2. The precision of the analysis of variance of the secondary responses could be increased by covariance correction for the primary responses. This has not been carried out since it would not modify interpretation of the data.

Table 2

ANALYSES OF VARIANCE OF THE DATA IN TABLE 1

Source of Variation	Primary Response		Secondary Response	
	D.F.	Mean Square	D.F.	Mean Square
Doses	(2)		(3)	
Linear regression	1	258***	1	200***
Deviations	1	1	2	7
Times	4	64	4	43
Doses × times	(8)		(12)	
Linear × times	4	13***	4	18**
Remainder	4	2	8	6
Error	60	3·35	80	5·11

** $0.01 > P > 0.001$.

*** $P < 0.001$.

(b) *Study of Titre Measurement*

There are a number of different methods for determining haemolytic titre. The end-point used above was described in Sobey and Adams (1955*a*). Briefly this is an interpolative method in which the titre corresponding to an arbitrary haemolysis level midway between complete and no haemolysis is estimated. The following is a further study of this end-point:

(i) *Discriminant Analysis.*—Fisher (1954) gives an account of the use of discriminant analysis in deriving additive scores for arbitrary serological readings. This method has been used to estimate scores for the readings 1, 2, 3, 4 of incomplete haemolysis judged visually which are assumed intermediate between reading 0 (no haemolysis) and reading 5 (complete haemolysis). The results of a series of 96 titre determinations were available for analysis in this manner, and an additive score was determined which maximized differences between dilutions compared with the experimental error. Using this additive score, 82 per cent. of the total variation of the 96 determinations was accounted for by differences between dilutions. Corresponding with the series of arbitrary response levels (5, 4, 3, 2, 1, 0) the estimated additive levels were 5, 3·7, 3·1, 2·5, 1·7, 0 respectively. While the estimated score agrees closely with the arbitrary score at the region of greatest haemolysis it diverges from the arbitrary score elsewhere. This divergence is statistically significant. Use of the arbitrary score in preference to the estimated score leads to a small bias in each titre estimate. Since such a bias is always in the same direction and since an absolute estimate of response is unnecessary the arbitrary scores were subsequently used for convenience.

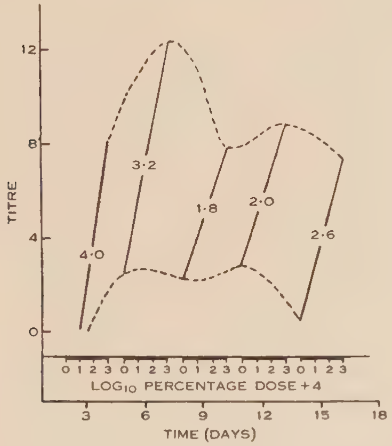

Fig. 1

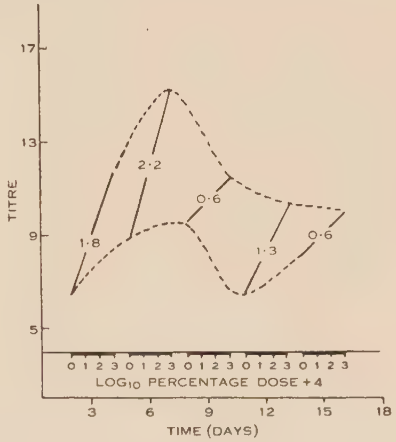

Fig. 2

Fig. 1.—Relation of primary response to log dose and time. Broken lines indicate the time-response curves obtained with 0·1 and 0·0001 per cent. sheep red cells. Dose-response slope is indicated on each dose-response time.

Fig. 2.—Relation of secondary response to log dose and time. Broken lines indicate the time-response curves obtained with 0·1 and 0·0001 per cent. sheep red cells. Dose-response slope is indicated on each dose-response time.

(ii) *Haemoscope Red Cell Estimation.*—An experiment was carried out to determine the relationship between antibody level and the number of unhaemolysed red cells in the tubes from 5 to 0. A number of similar dilution series were set up and the antibody levels scored. Ten samples at each antibody level (i.e. 0,1, ... 5) were chosen at random from these series. Two million red cells were added to each tube to bring the cell number on to the scale of the haemoscope (Sobey and Adams 1955*b*). A further 10 tubes were set up containing 2 million cells in normal saline. Standard samples were taken from each tube after shaking and were counted in random order by the haemoscope. The means of cell counts may be seen in Figure 3. The standard error of each mean count is 0·069 haemoscope units. It is obvious (and this may be confirmed by analysis of variance) that there are no significant differences between the mean cell counts in the control tubes, and those scored as 5, 4, and 3. This means that the antibody levels in tubes scored as 5, 4, and 3 are visually discriminated by the size of the deposit of cell debris, and not by opalescence due to unhaemolysed red cells.

The relationship of the number of unhaemolysed red cells, and the corresponding visual score of antibody level at different dilution levels of antiserum was investigated in another experiment. Antiserum was diluted to six different levels and 10 replicates of each dilution were set up. These were first treated in the standard manner so that each tube received a fixed amount of complement and sheep red cells. They were then scored on the visual 0-5 scale in random order. Two million red cells were then added to each tube (as above) and the number of red cells estimated with the haemoscope. The mean values for the 10 tubes at each dilution were calculated for both measurements and are plotted in Figure 4. It may be seen that a change in the visual score occurs continuously with change of level of antiserum. However, the number of unhaemolysed red cells, as estimated by the haemoscope remains constant over the first four levels and then rapidly changes. Analysis of variance confirms both of these findings.

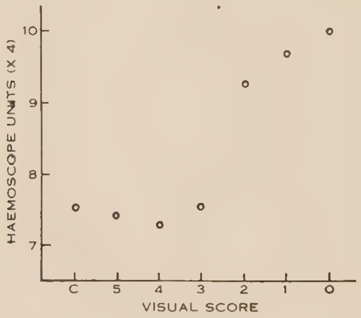

Fig. 3

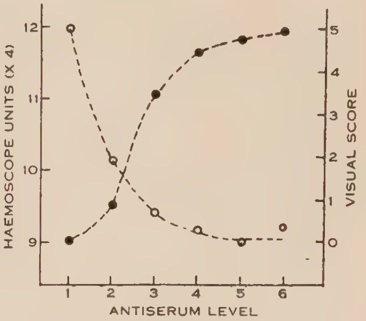

Fig. 4

Fig. 3.—Number of unhaemolysed red cells in tubes of a dilution series scored visually. *C* denotes the control described in the text. The standard error of each mean value plotted is 0·3 haemoscope units (×4).

Fig. 4.—Relation of both visual score (●) and number of unhaemolysed red cells (○) to antiserum level. The broken lines give the simplest description of the observations and were obtained by analysis of variance.

(iii) *Spectrophotometric Haemoglobin Estimations*.—The relationship of the amount of haemoglobin present in the supernatant liquid to the visual score at different levels of dilution was studied in this experiment. Twelve replicates of eight different serum dilutions were set up. After visual scoring, 0·3 ml of the supernatant fluid was placed in the semi-microcell of a "Uvispek" spectrophotometer and the optical density measured at a wavelength of 450 mμ. The solutions were held in a refrigerator prior to reading in order to eliminate bias due to time trends in haemoglobin concentration, and were then measured in a random order. Mean values of both scores are illustrated in Figure 5 where it is apparent that the spectrophotometer readings are running in parallel with the visual score. This was verified by analysis of variance.

III. Factors Influencing The Level Of Haemolytic Titre

Two experiments were carried out in this section for the purposes of generalizing some of the results obtained in the first paper of this series (Sobey and Adams 1955*a*).

(*a*) *Biological Variation*

Twelve adult domestic rabbits and 12 sheep were used in this experiment. Each rabbit received 4 ml of a 1 per cent. suspension of sheep red cells in two injections, 1 week apart. The sheep and rabbits were randomly numbered from 1 to 12. Red cells obtained from the sheep numbered 1 were given to the rabbit numbered 1 and so on. The rabbits were bled 1 week after the second injection and the sera separated in the standard manner.

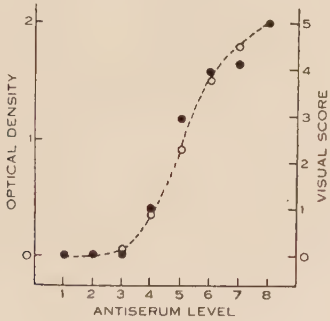

Fig. 5

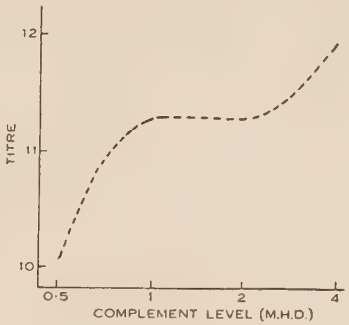

Fig. 6

Fig. 5.—Relation of visual score (●) and optical density of the supernatant (○) to antiserum level. The broken line fits both sets of observations, and was obtained by analysis of variance.

Fig. 6.—Effect of complement level on titre. The broken line gives the simplest description of the experimental data.

Each serum was titrated against red cells obtained from every sheep so that in all 144 titrations were carried out. Twelve titrations were thus made with homologous material and 132 with heterologous material. The experimental data are given in Table 3, and the analysis of variance in Table 4.

The analysis indicates that both source of sheep red cells and source of rabbit antiserum have a marked influence on the titre obtained. It is evident that the titre is not significantly influenced by heterologous red cells. Thus if a particular sheep's red cells have a low titre with a given antiserum, they will give low titres with all other antisera.

(*b*) *Measurement Variation*

In this experiment many of the factors which are thought to influence haemolytic titre were varied simultaneously. These were (i) age of complement, (ii) age of sheep red cells, (iii) level of complement, (iv) time of reading titre, and (v) time of day of carrying out experiment. In a preliminary experiment for the purposes of determining the minimal haemolytic dose (M.H.D.) of complement two rabbit antisera were used, one of low titre and another of high titre. Since similar M.H.D.'s were found with both antisera this variable was not included in the experiment.

Complement was obtained by cardiac puncture from 20 adult male guinea pigs, pooled, and stored in glass ampoules at −20° C for 6 weeks. On the day of the experiment a further supply of complement was obtained and this was compared with the stored complement. Red cells were obtained from a sheep 4 days before the experiment and stored in a refrigerator at 4° C. These were compared with red cells obtained from the same sheep on the day of the experiment. Four levels of complement were used in this experiment: 4, 2, 1, and ½ M.H.D. The experiment was carried out in two replicates, the work of the first replicate being completed before the second replicate was begun. The experiment was also replicated over three sources of rabbit antisera so that the design is a full $2^3 \times 3 \times 4$ factorial experiment (Fisher 1955).

TABLE 3

TITRES (×4) OBTAINED IN THE STUDY OF BIOLOGICAL VARIATION. TITRES OBTAINED WITH HOMOLOGOUS RED CELLS ARE ITALICIZED

Rabbit No.	Sheep No.											
	1	2	3	4	5	6	7	8	9	10	11	12
1	*52*	55	52	50	53	51	58	52	50	53	53	55
2	62	*59*	56	56	52	54	58	52	55	45	56	70
3	70	61	*59*	61	60	56	62	56	55	60	60	64
4	64	55	54	*56*	56	60	58	61	56	45	62	67
5	72	56	64	60	*61*	64	63	64	63	59	64	69
6	59	54	56	50	53	*58*	61	48	57	51	59	60
7	56	52	55	50	50	50	*52*	49	49	52	55	59
8	55	52	56	53	54	50	58	*50*	51	53	54	55
9	58	60	54	54	55	52	59	50	*54*	56	55	66
10	62	58	56	52	58	50	59	60	56	*59*	59	70
11	58	48	51	47	48	46	51	58	50	44	*53*	52
12	58	56	57	54	57	51	60	53	54	55	55	*70*

Titres were read at random ½ hr after incubation at 37° C for ½ hr, and again (the same tubes) after overnight refrigeration at 4° C. Titres are given in Table 5 and the analyses of variance in Table 6. Since each titration has been scored on two occasions, these pairs of titres will be highly correlated and it is invalid to include this variable in a simple analysis of variance. A simple method of overcoming this difficulty is to make an analysis of variance of the titres after incubation, and a separate analysis of variance of the overnight change in titre as measured by the difference of the immediate titre and the titre after refrigeration overnight.

All variables studied cause variation in the titre determined immediately after incubation (T_1). Since no significant interaction is found at this time, the total effect of the variables is the sum of their individual independent effects. Thus provided any comparison between antisera is made with some arbitrarily selected combinations of these variables, consistent results can be expected. The titres obtained after overnight refrigeration are, on the average, higher than those obtained after incubation. Analysis of the overnight difference ($T_1 - T_2$) shows this to be quite large (0·83 of a titre unit) but constant over all experimental variables other than age of complement. With stored complement the average titre increased by $0·61 \pm 0·03$ units overnight, while with fresh complement the titre increased by $1·04 \pm 0·03$ units overnight.

TABLE 4

ANALYSIS OF VARIANCE OF THE DATA IN TABLE 3

Source of Variation	D.F.	Mean Square
Rabbits	11	10·33***
Sheep	11	7·18***
Homologous × heterologous	1	0·36
Remaining interactions as error	120	0·531

*** $P < 0·001$.

The variation in titre due to different levels of complement may be further analysed in linear, quadratic, and cubic components. This was not included in the analysis of variance since each component was highly significant, indicating a complex titre-complement level relationship (see Fig. 6).

(c) *Components of Variation*

It is reasonable to assume that the variances estimated above will be constant for the randomly bred animals and for the standard techniques employed. We may combine the results of the experiments to arrive at estimates of the components of variation. A titre can be supposed to be the sum of an effect due to a particular animal, a constant fixed by the experimental technique, and an error due to inaccuracy in measurement. If these contributions are independent, the total variance of antibody responses in a population of individuals is the sum of the variance of individuals plus the variance due to measurement errors. Thus $V_T = V_B + V_M$, where V_T is total variance, V_B is variance of individuals, and V_M is measurement variance.

The second experiment above (Table 6) gives an estimate of $V_M = 0·065 \pm 0·011$, while the first (Table 4) may be used to obtain an estimate of V_B. The interactions in this analysis include a contribution due to measurement variation. Removing

TABLE 5

TITRES (× 4) OBTAINED IN THE STUDY OF MEASUREMENT VARIATION

Incubation Period (see text)	Antiserum No.	Level of Complement (M.H.D.)	Replicate 1				Replicate 2			
			Stored Cells		Fresh Cells		Stored Cells		Fresh Cells	
			Stored Complement	Fresh Complement	Stored Complement	Fresh Complement	Stored Complement	Fresh Complement	Stored Complement	Fresh Complement
T_1	1	4	44	47	45	44	40	42	43	42
		2	35	42	44	43	35	37	40	38
		1	34	42	44	43	31	39	37	41
		½	39	33	38	40	33	41	36	39
T_1	2	4	45	51	48	47	43	45	48	46
		2	45	46	45	52	36	46	44	49
		1	48	47	47	48	36	42	44	45
		½	42	36	36	45	36	44	38	34
T_1	3	4	47	55	59	56	51	50	52	54
		2	52	53	56	54	36	52	46	55
		1	51	48	50	56	47	51	58	54
		½	39	40	48	48	39	52	47	43
T_2	1	4	46	50	45	52	43	45	43	46
		2	36	48	44	46	38	44	40	44
		1	36	45	46	44	35	45	38	44
		½	39	36	36	43	36	46	38	55
T_2	2	4	47	54	48	52	51	49	52	51
		2	48	52	47	53	38	50	43	50
		1	45	48	47	48	33	44	44	50
		½	44	46	45	46	36	46	38	43
T_2	3	4	53	60	61	58	56	54	56	55
		2	59	56	58	56	41	58	48	57
		1	55	57	57	58	48	56	60	57
		½	52	44	48	55	41	52	56	47

this it is found that the variance between rabbits is $(10{\cdot}33-0{\cdot}53)/11 = 0{\cdot}89 \pm 0{\cdot}12$ which may be taken as V_B.
Therefore

$$0{\cdot}955 = 0{\cdot}890+0{\cdot}065,$$

or

$$100\% = 93\%+7\%.$$

It may immediately be deduced that the correlation between replicate estimates of the haemolytic titre is 0·93, a value consistent with that given in the first paper in this series (Sobey and Adams 1955*a*) which was obtained by direct computation of the correlation. Thus the measurement variation is a very minor component in the variation of haemolytic titres.

TABLE 6

ANALYSIS OF VARIANCE OF THE DATA IN TABLE 5

Source of Variation	D.F.	Mean Square	
		T_1	T_1-T_2
Mean†	1	—	65·84***
Replicates	1	0·94***	0·03
Age of complement	1	0·94***	0·44**
Age of sheep red cells	1	1·32***	0·07
Level of complement	3	14·44***	0·09
Source of antiserum	2	5·29***	0·13
First order interaction	24	0·092	—‡
Error	64	0·0652	
	87		0·0545

** $0{\cdot}01 > P > 0{\cdot}001$.

*** $P < 0{\cdot}001$.

† The mean of the observations is usually assumed to be significantly different from zero, and the effect of this removed by the correction factor in the analysis of variance. In the analysis of a difference we are interested in testing whether the mean difference is zero and we thus include this item.

‡ Since analysis of variance of (T_1-T_2) is effectively an analysis of the interaction between time of reading titres and other variables, the first order interactions of (T_1-T_2) have not been examined.

IV. DISCUSSION

The biphasic time-response curves obtained with both primary and secondary responses may be explained in either of two ways. It may be suggested that different sites of antibody production have different times of maximum production of antibody. On the basis of the present work it would seem that one set of sites could be involved in the immediate production of antibody and another set with a more delayed production of antibody. Alternatively, it may be suggested that the biphasic curve is the sum of two (or more) monophasic response curves each of which corresponds to a single antigen of the red cell.

On the basis of published evidence the complex titre-complement level relationship was quite unexpected. This relationship may be divided into three regions (i) a central region in which titre does not depend on complement level, and (ii) two extreme regions in which increasing complement level is associated with increased titre.

In the central zone the amount of haemolysis is simply a function of the relative concentration of antibody and antigen, while in the extreme zones haemolysis is also a function of complement level. The M.H.D. unit used in the investigation is larger than that commonly used by others. Our M.H.D. is estimated by a checkerboard arrangement of different dilutions of antisera combined with different dilutions of complement. Using this system over numerous tests of fresh guinea pig serum we find that fresh serum is equivalent to 8-16 M.H.D.'s.

It must be stressed, however, that the level of complement used in a test may be chosen arbitrarily provided the level is kept the same for all comparisons, since we have found the titres additive over all levels of complement used. In fact, since it has been found that many variables in haemolytic titre estimations are capable of such arbitrary definition, it appears that many of the arguments in the past about these variables are quite pointless. Any haphazard changes of technique must be avoided since even minor changes can increase variability and thus reduce accuracy. Appropriate experimental designs enable variation due to alteration in technique to be excluded from the experimental errors with resultant maximum accuracy.

ACKNOWLEDGMENTS

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REFERENCES

FISHER, R. A. (1954).—"Statistical Methods for Research Workers." 12th Ed. (Oliver and Boyd: Edinburgh.)

FISHER, R. A. (1955).—"The Design of Experiments." 6th Ed. (Oliver and Boyd: Edinburgh.)

MATHER, K. (1949).—"Biometrical Genetics." (Methuen: London.)

SANG, J. H., and SOBEY, W. R. (1954).—*J. Immunol.* **72**: 52.

SOBEY, W. R. (1954).—*Aust. J. Biol. Sci.* **7**: 111.

SOBEY, W. R., and ADAMS, K. M. (1955*a*).—*Aust. J. Biol. Sci.* **8**: 603.

SOBEY, W. R., and ADAMS, K. M. (1955*b*).—*Med. J. Aust.* **1**: 527.

CONDUCTANCE CHANGES AND RESPONSES IN POTATO TUBERS FOLLOWING INFECTION WITH VARIOUS STRAINS OF *PHYTOPHTHORA* AND WITH *PYTHIUM**

By C. G. Greenham† and K. O. Müller†

[*Manuscript received October* 24, 1955]

Summary

Electrical conductance measurements were made at high frequency (1 Mc/s) and at low frequency (1 kc/s). The high frequency (H.F.) conductance values were similar in healthy and diseased tissue; the low frequency (L.F.) conductance values increased with duration of infection, and changes in them could be used as a quantitative measure of injury.

In 1–2 days a parabiotic combination of *Phytophthora infestans* De Bary and potato tuber results in injury to the infected face, whereas a eusymbiotic combination results in no measurable injury. Four days after inoculation, in the parabiotic combination injury extends at least 4 mm in advance of the pathogen, whereas in the eusymbiotic combination there is no measurable injury in freshly-invaded tissue.

Conductance changes as measured are not suitable for determining the highest degree of clinical resistance, due to rapid hindrance of the growth of the pathogen (local lesion reaction).

Conductance measurements on tubers invaded by *Pythium* sp. show that the pathogen causes injury some millimetres behind the hyphal tips.

The results are discussed with reference to (i) the rate of development of injury, (ii) the nature of the injury caused, and (iii), in the parabiotic combination, the liberation of a principle rendering the host cells inhospitable to the advancing pathogen.

I. INTRODUCTION

Injury and host response following infection are of fundamental importance in studies on host-pathogen relationships. Such studies have led a number of phytopathologists to regard supra-sensitivity as an essential factor in the basis of host resistance to some diseases, e.g. rust and smut of cereals, or late blight and wart of potatoes. For such diseases, a parasitic relationship can be established only if the host cell "tolerates" the pathogen for a certain period of time. When interaction in a supra-sensitive fashion occurs, however, hastened necrosis of the host tissue occurs and growth of the parasite ceases, i.e. the host is clinically resistant. Thus for potato tubers infected with *Phytophthora infestans*, Müller and Börger (1939) and Müller (1953) suggested that a parasitic relationship is established only if there is "concordance" between the metabolic systems of both the host and parasite. If the systems are not compatible during the initial infection, the physiological unity of the host cell breaks down and some activated principle checks the spread of the pathogen. Subsequently Gäumann (1950) coined the terms "eusymbiotic"* and "parabiotic"* to designate the type of host-pathogen relationship, and Müller (1953) propounded the paradoxical idea that the less the primary injury caused by infection, the greater is the susceptibility of the host.

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Injury resulting from infection has been measured by means of vital stains, plasmolysis, and other methods (Strugger 1936; Meyer 1939). From the classical works of Osterhout (1922) and Höber (1945) it was known that at low frequency living tissues have low conductance,† which may be increased by the action of poisons. This paper describes experiments in which conductance of the potato tuber is used as a precise and quantitative measure of injury following infection. Most of the experiments deal with infections caused by certain strains of *Phytophthora infestans* De Bary which are responsible for eusymbiotic or parabiotic types of infection. In addition, a few experiments deal with *Pythium* sp. (*P. ultimum* according to British authors).

II. Material and Methods

Potato tubers were inoculated with *Phytophthora* by the application of a disc of filter paper, dipped in a suspension of zoospores, to a freshly-cut surface. The tubers, with infected surface uppermost, were rested on glass slides in a dish lined with damp blotting-paper and covered with a sheet of glass. Control tubers or half tubers were similarly treated except that the disc of filter paper was dipped in distilled water. The dishes were left for one or more days at 20°C.

Table 1

INTERACTION BETWEEN POTATO VARIETY AND PHYTOPHTHORA STRAINS A, B, AND C

Host	*Phytophthora* Strain:		
	A	B	C
Factor (Up-to-Date)	Eusymbiotic	Eusymbiotic	Eusymbiotic
Late Carmen	Eusymbiotic	Eusymbiotic	Eusymbiotic
Kennebec	Parabiotic	Eusymbiotic	Parabiotic

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* In a eusymbiotic combination, the host initially shows a mild reaction and tolerates the parasite for a limited period; in a parabiotic combination, the host reacts strongly, does not tolerate the parasite, and its infected cells soon die. The resistance or susceptibility of the host may depend on both the host and pathogen. Thus variety A of potato can be resistant to strain X of *Phytophthora infestans* and susceptible to strain Y; conversely variety B can be resistant to strain Y and susceptible to strain X.

† The term "conductance", which is the reciprocal of electrical resistance, is now preferred to the former "conductivity". Osterhout, while using the term conductivity in the title of his monograph, plotted his results in terms of electrical resistance. Elsewhere in this paper, unless stated otherwise, the term "resistance" is used only to indicate the clinical result of interaction of the host with the pathogen.

For the main experiments the potato varieties and international strains of *Phytophthora infestans* shown in Table 1 were used. The interaction between these strains and their hosts is also shown by the table.

With Kennebec and *Phytophthora* strains A and C the parabiotic reaction is due to the presence of gene R_1 (Müller 1930; Black 1952), but the reaction does not represent the highest degree of supra-sensitivity. The parasite usually penetrates 5–7 mm into the tuber but it is then checked.

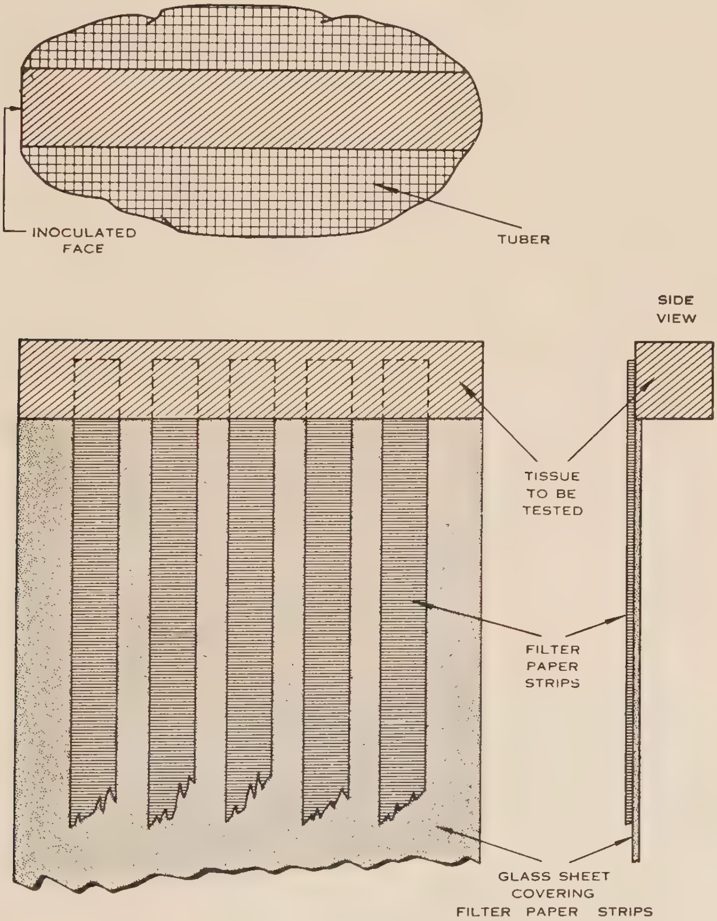

Fig. 1.—Diagram showing use of filter paper strips to determine amount of liquid exuded.

The variety Sebago was mainly used in the *Pythium* experiments. To induce infection, a freshly-cut face on a piece of tuber was placed on a potato-dextrose-agar culture on a glass slide in a dish of the type described above. This avoided any cell exudate trickling down over healthy portions of the tuber. To measure the exudation of water caused by *Pythium*, pieces of coloured filter paper were placed on a surface cut at right angles to the infected surface; degree of water exudation was determined by the distance for which water soaked along the pieces of filter paper (see Fig. 1).

Presence or absence of infection was determined by transferring explants aseptically to cuts in healthy susceptible tubers, and noting presence or absence of developing pathogen after 5 days in a humid atmosphere (20°C). For the slice technique, the explants were taken from the wall of the hole left after the removal of the plug (see below).

Low frequency (L.F.) conductance (1 kc/s) was measured by means of the simple A.C. bridge previously described (Greenham and Cole 1950), the meter being calibrated in mhos (reciprocal ohms). Conductance was measured on slices immersed in 0·6 per cent. sea-salt solution (Greenham 1952), or by means of two metallic probe tips (Greenham and Cole 1950). The discs, 1 mm thick and 6 mm diameter, were sliced by means of a hand microtome from a cylinder cut with its axis at right angles to the inoculated face; each disc was kept in a separate beaker of saline solution, and was measured after immersion for 15–30 min. One or two slices nearest to the infected faces were discarded to exclude desiccated or contaminated tissue, either of which can give abnormal conductance readings. Bacterial contamination, not readily avoided, frequently occurs on faces infected with a eusymbiotic pathogen. Desiccation, due to rapid death of the host cells, often results from a parabiotic combination, and then there is little bacterial contamination.

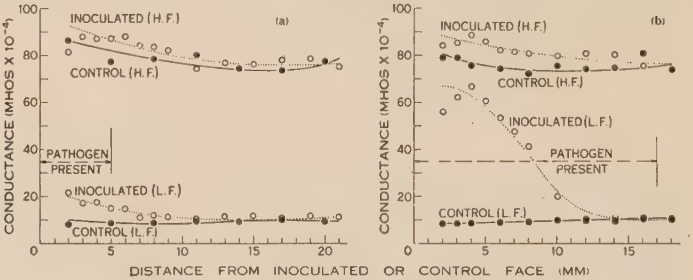

Fig. 2.—Conductance measurements (in saline solution) on discs of potato tuber inoculated with *P. infestans*; (*a*) parabiotic combination (Kennebec-strain C), (*b*) eusymbiotic combination (Kennebec-strain B). The readings are plotted as obtained, i.e. some saline solution (conductance approx. $1{\cdot}25 \times 10^{-2}$ mhos) is in series with each disc. 2 mm distance represents disc at 1–2 mm from inoculated face, and so on.

The probe readings, with needles at 3·5 mm separation, were made either on the inoculated face or on a surface cut at right angles to the inoculated face. When the latter method was used, the following operations were carried out. A face was cut on a healthy tuber, then the tuber was cut at right angles to the exposed face, to give two equal pieces. The initial faces of the pieces were treated with filter paper or agar. A few days later a slice 2 mm thick was removed at right angles to the inoculated or control faces of the pieces and discarded. The freshly-exposed faces were dried with filter paper, and probe readings were made on them, commencing in the healthy zone and finishing in the infected zone. As necessary, the probe tips were sterilized with 60 per cent. alcohol and dried in an air blast.

High frequency (H.F.) conductance (1 Mc/s) was determined by means of a Wayne-Kerr type B601 bridge, with electrical resistance and capacitance in parallel. The output from the bridge was fed into a selective amplifier with in-built beat frequency oscillator, the bridge balance being determined on a cathode ray oscillograph.

It should be noted that the L.F. probe readings can show a conductance gradient within a tuber. Accordingly, when comparing one cut half with another, the probe insertions were paired for position. In addition, the readings were treated statistically whenever possible.

III. Results

(*a*) *Experiments with* Phytophthora

(i) *Variation in Conductance with Distance from Infected Face.*—Figures 2(*a*) (parabiotic combination) and 2(*b*) (eusymbiotic combination) are typical of results obtained with the slice technique 4 days after inoculation. Figures 3(*a*) and 3(*b*) are examples of results obtained with the probe technique 4 days after inoculation, the measurements being made on a surface cut at right angles to the inoculated or control face. The probe readings are lower than the corresponding disc readings, due mainly to a longer path and smaller effective cross-sectional area for conduction in the tuber parenchyma. Nevertheless, the L.F. measurements with the probe are comparable with those made on the discs.

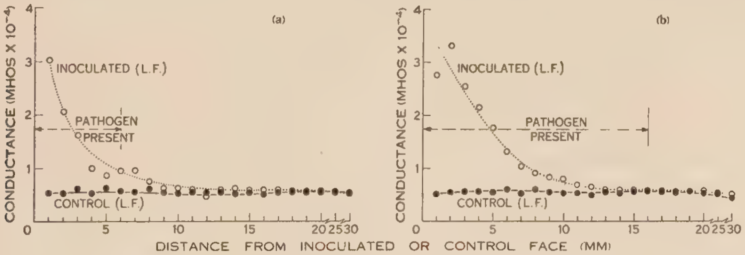

Fig. 3.—L.F. probe conductance measurements on portions of tubers inoculated with *P. infestans*; (*a*) parabiotic combination (Kennebec-strain C), (*b*) eusymbiotic combination (Factor-strain C).

The nature of the curves is such as to justify the use of conductance measurements for measuring the injury caused by the pathogens: L.F. conductance decreases with distance from the infected face (the longer the period for which the pathogen has been present, the greater is the injury (cf. Greenham and Cole 1950, Fig. 4)); L.F. conductance is constant with distance in control tissues and is much lower than H.F. conductance; H.F. conductance is approximately constant and the same in both control and experimental sections (Luyet (1932) showed that the conductance of live tissues approaches that of dead tissues); thus the difference between H.F. conductance and L.F. conductance increases with distance from the infected face. In fact, once the difference between the H.F. and L.F. measurements has been established, the L.F. measurements are enough to determine the injury caused by a pathogen, the L.F. readings being low and approximately constant in healthy tissues, and increasing with injury.

TABLE 2

DIFFERENCE IN LOW FREQUENCY CONDUCTANCE (IN UNIT MHOS $\times 10^{-6}$) BETWEEN INOCULATED AND HEALTHY TUBER HALVES (VAR. KENNEBEC)

Phytophthora Strain	Distance in Advance of Pathogen (mm):									
	1	2	3	4	5	6	7	8	9	10
Strain C										
Inoculated—control (12 replications)	+9·67***	+10·25***	+7·66**	+6·66**	+4·34 (n.s.)	+2·75 (n.s.)	+3·92 (n.s.)	+3·91 (n.s.)	+3·42 (n.s.)	+2·33 (n.s.)
Strain B										
Inoculated—control (11 replications)	−1·46 (n.s.)	−2·45 (n.s.)	−2·28 (n.s.)	−2·00 (n.s.)	+1·50 (n.s.)	+1·50 (n.s.)	+4·50 (n.s.)	+3·50 (n.s.)		

** $P < 0{\cdot}01$. *** $P < 0{\cdot}001$. n.s. = Not significant.

The conductance of the pathogen has no serious effect on that of the host tissue: particularly at L.F. with the probe technique, the conductance of host tissue plus conductance of pathogen would tend to be higher than conductance of the host tissue alone, due to two conductors being in parallel. However, as is shown below and also in Section III (*a*) (iii) for the eusymbiotic combination, the initial presence of the pathogen does not affect the L.F. conductance of the host tissue. The great increase in L.F. conductance in the first infected tissue is far more than is warranted by the presence of another poor conductor, viz. the pathogen (the mycelium of the pathogen behaves like the host tissue, in that its conductance is much greater at H.F. than at L.F.).

Accordingly, it may be concluded that L.F. conductance of the tuber tissues, as determined either with the disc or probe technique, is satisfactory for the quantitative measurement of injury caused by the pathogens.

From Figures 2 and 3 it is seen that in the eusymbiotic combination the conductance of freshly-invaded tissue is the same as that of healthy tissue, so it is concluded that the pathogen can temporarily co-exist with the host without causing measurable injury. It is also seen that in the parabiotic combination the conductance of freshly-invaded tissue is greater than that of distant healthy tissue, so it is concluded that for this type of combination the pathogen cannot co-exist with the host without causing injury. These observations have been repeated on numerous occasions and with other pathogen strains and host varieties.

(ii) *Injury in Advance.*—An examination of the infected and control L.F. curves in Figures 2 and 3 suggests that there is injury in advance of the pathogen when the combination is parabiotic, but not when it is eusymbiotic. This suggestion was checked in the following manner:

Some Kennebec tubers were cut into halves, the cut ends being inoculated with either strain B or strain C or being treated as controls. After 4 days, L.F. measurements were made on freshly-exposed surfaces cut at right angles to the inoculated or control ends. The probe tips were 2 mm long. Presence or absence of pathogen was also determined. For the statistical analyses, the differences in conductance readings between the infected and control pieces were taken at the same distance from the inoculated or control faces (Table 2).

From the conductance differences in Table 2 it is seen that in the parabiotic combination the pathogen has a significant effect 4 mm in advance; the effect probably extends further, though the data are not sufficiently numerous to make it significant. For the eusymbiotic combination, there is no evidence of an effect in advance.

(iii) *Clinical Response to Infection and Rate of Development of Injury.*—The following experiment was carried out to test the theory that the infected cells are injured more quickly in a parabiotic combination than those in a eusymbiotic combination: Kennebec tubers were cut in half; the matched halves were inoculated or treated with distilled water on a central area 2×2 cm delimited with four ink dots. Twenty-six hr later, measurements were made on half of each of the inoculated or control area; 24 hr later, measurements were made on the remaining halves of the selected areas. Table 3 summarizes the results.

The differences between the means give excellent support to the theses (1) the host tissue shows rapid injury when the parabiotic combination occurs, (2) the host tissue initially tolerates infection when the eusymbiotic combination occurs. On the whole there is very little difference between the means for strain B and the means for the controls.

TABLE 3

MEAN LOW FREQUENCY CONDUCTANCE READINGS (IN UNIT MHOS × 10^{-5}) ON INOCULATED OR CONTROL AREAS (KENNEBEC TUBERS)

Values given are the means of 9 readings. Probe tips 1·5 mm long

Time after Inoculation (hr)	*Phytophthora* Strain A (parabiotic)	*Phytophthora* Strain B (eusymbiotic)	Control	Difference Required at $P = 0{\cdot}05$	Statistical Significance of Difference
26	4·56	4·02		0·35	$P < 0{\cdot}01$
	4·61	4·03		0·20	$P < 0{\cdot}001$
		3·83	3·80	0·10	n.s.
		3·86	3·74	0·14	n.s.
	4·76		3·93	0·33	$P < 0{\cdot}001$
	5·38		4·16	0·20	$P < 0{\cdot}001$
50	7·81	5·44		1·49	$P < 0{\cdot}01$
	6·23	4·56		0·49	$P < 0{\cdot}001$
		4·40	4·36	0·26	n.s.
		4·16	4·08	0·34	n.s.
	6·00		4·35	0·71	$P < 0{\cdot}001$
	6·00		4·29	0·39	$P < 0{\cdot}001$

In a second experiment similar results were obtained on the first and second days after inoculation, and there were no significant differences between paired non-infected faces. On the third and fourth days, the L.F. conductance readings on the faces infected with strain A were less than those on the faces infected with strain B. This reversal of difference is attributed to the development of bacteria with strain B (visible on the third and fourth days) and to desiccation of the outer cells infected with strain A. In this experiment such desiccation was shown by means of additional H.F. conductance readings on the fourth day. The H.F. readings on faces infected with strain A were significantly less than those on the faces infected with strain B (cf. Greenham and Cole 1950, Fig. 9).

The desiccation of cells infected with strain A is also demonstrable clinically. The cells lose their turgidity rapidly, so that small pits develop at the loci of infection. In both the above experiments, the dying or dead cells were observed by the naked eye as brown pits, slightly visible after 24 hr, readily visible after 48 hr.

An assessment of the period required for the development of injury in a eusymbiotic combination was also obtained from an examination of conductance–distance graphs as shown in Figure 3(*b*). From an estimate of the length of the asymptotic portion of the curve, within the infected zone, of 13 such graphs, and from a knowledge of the rate of penetration of the pathogen, it was calculated that, on the average, some 38 hr elapse before there is noticeable change in conductance. This value agrees reasonably well with that of over 50 hr indicated by Table 3. This agreement contradicts the possible suggestion that lack of change in conductance on the faces infected with the eusymbiotic combination (Table 3) is caused by antagonism resulting from the frequent surface contamination with bacteria.

(iv) *The Use of Conductance Measurements for Determining Degree of Supra-sensitivity.*—In view of the results reported in Section III (*a*) (iii), it was considered that when supra-sensitivity occurred, conductance values would change more rapidly in the inoculated zone than in the surrounding area. The following experiment was therefore carried out.

TABLE 4

CORRELATION TABLE FOR DIFFERENCE IN CONDUCTANCE AGAINST CLINICAL GROUPING

Clinical Rating*	Difference in Conductance (mhos $\times 10^{-5}$) (mean of inner zone minus mean of outer zone)							
	−0·15	0	0·15	0·30	0·45	0·60	0·75	0·90
	No. of Tuber Halves in Categories I–IV							
I†	1	1	5	4				
II†			7	4	0	2	0	1
III†		2	2	0	2	1		
IV†	8	21	19	2				

* Rating I indicates the highest degree of biological resistance, rating IV the lowest.

† Rating I, local lesions only; rating II, penetration to 3 mm, no fructifications developed; rating III, large parts of the tuber penetrated, sparse fructifications; rating IV, tuber completely penetrated, extensive fructifications.

Tuber halves from 14 different varieties of seedlings were inoculated in a central area, as in Section III (*a*) (iii), but with strains A, B, or C. L.F. readings were made 48 hr later with probe tips 1·5 mm long, both in the central zone and the zone 3–7 mm outside. Concurrently, an estimation of the rapidity of injury was made by eye, on the basis of clinical symptoms (ratings I to IV). The results are summarized in Table 4, the values in the table indicating the number of tuber halves occurring in the appropriate categories.

It is seen that groups IV, III, and II, representing increasing reactive resistance, show increasing conductance differences. However, group I, representing the group with the greatest reactive resistance, does not fall within such regression, suggesting response of a different type. The explanation for this is that in group I the infected cells die rapidly and do not transmit infection; accordingly the conductance test *as used* is not satisfactory for selecting individual tubers showing the highest degree of biological resistance.

(b) *Experiments with* Pythium

The injury caused by *Pythium*, in contrast with that caused by *Phytophthora*, results in a considerable exudation of liquid from the tubers, hence the popular name "watery wound rot". However, there are further differences between the two pathogens: (1) *Phytophthora* contacts the host plasm directly by haustoria; these are developed by the hyphae which fill the intercellular spaces and penetrate the cell walls. Obviously they are the organs through which certain constituents of the host cells are translocated to the intercellular hyphae. The hyphae of *Pythium* are as a rule restricted to the intercellular spaces, but develop no true haustoria. There is no doubt that this parasite's nutritional requirements are largely supplied by the liquid which is released by the injured host cells into the intercellular spaces. (2) Apart from loss of turgidity by release of water, the parasitized host tissue does not show any of the specific morphological changes as observed in tubers diseased with *Phytophthora* (Meyer 1939), e.g. decomposition of starch grains or increased affinity to vital strains, which would indicate changes in the physiological activity of the affected host cells. Thus there is good reason to assume that two essentially different mechanisms of host-pathogen relationship are involved.

TABLE 5

RELATIVE AMOUNTS OF CELL SAP RELEASED BY TUBER (VAR. KING EDWARD) TISSUES INFECTED WITH PYTHIUM AND PHYTOPHTHORA* RESPECTIVELY

Incubation period 5 days, temperature 15–21°C. The figures represent the length (mm) of filter paper strips soaked with cell sap

Region (distance from infected face)	*Pythium*			
	Exposure Periods (hr):			
	2	19	26	43
1 (1–6 mm)	29	83	103	174
2 (12–17 mm)	22	72	75	120
3 (22–27 mm)	0	9	20	87
4 (32–37 mm)	0	0	0	11
5 (42–47 mm)	0	0	0	0

* Similar exposure periods and regions investigated for control and *Phytophthora*-infected tubers—every reading zero.

(i) *Exudation of Liquid from Infected Cells.*—The degree of injury caused by *Pythium* infection was measured by the filter paper method described in Section II (by one of the authors (K.O.M.) whilst at the National Institute of Agricultural

Botany, Cambridge). As controls, healthy tissue and tissue infected by *Phytophthora* (eusymbiotic combination) were used. Five regions at different distances from the inoculated face were investigated. At the end of the test the tissues of the different regions were examined for presence or absence of the pathogen. In the case of *Pythium* the pathogen was isolated from regions 1–4 and in the case of *Phytophthora* from regions 1–3.

The results of such an experiment are given in Table 5. It will be seen that no measurable amount of liquid was released by control tissues or those infected by *Phytophthora*. In *Pythium* infected tissues, however, the exudation of cell sap was considerable; the longer the period of infection, the greater was the exudation of cell sap.

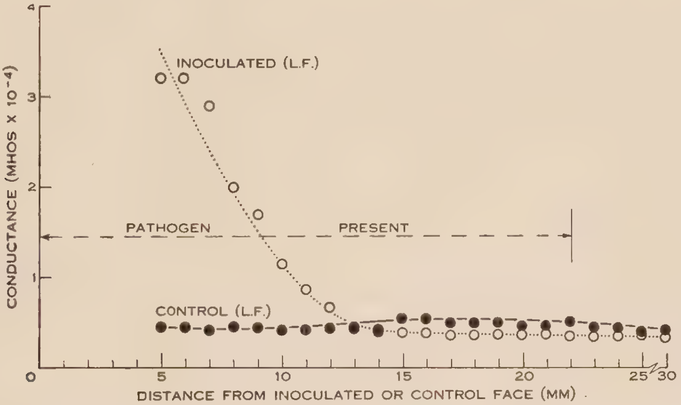

Fig. 4.—L.F. probe conductance measurements on tuber inoculated with *Pythium* sp.

(ii) *Conductance of Infected Tissue.*—The above method does not show the precise location of injury in relation to the hyphal tips. The conductance method was used to investigate this.

Preliminary experiments with tuber slices gave results comparable with those shown in Figure 2(*b*). The L.F. conductance of infected tuber halves was studied in more detail with the probe technique, 3 days after inoculation. Figure 4 is typical of results obtained on a surface cut at right angles to the inoculated face. It is seen that the pathogen can temporarily co-exist with the host without causing change in conductance.

From Figure 4 it might be thought that no conductance changes would occur within a day on a freshly inoculated face. Accordingly an experiment similar to that described in Section III (*a*) (iii) was carried out. After 24 hr each L.F. conductance reading on the inoculated areas was approximately twice the corresponding reading on the control areas (difference between areas significant at $P < 0{\cdot}001$). The probe tips were 1·6 mm long, so explants were taken after the conductance measurements, at 2–3 mm and 3–5 mm distance from the infected faces. Almost invariably, infection was found in the former explants, and in some instances in the latter explants, showing that the pathogen was usually present beyond the main conduction zone. Moreover, injury need not occur as deeply as the probe tips penetrate for there to be an increase in conductance around the probe tips. Therefore this experiment in no way contradicts the claim that *Pythium* can temporarily co-exist with the host without causing measurable injury. In addition, there may have been toxins present in the infected agar (Brandenburg (1948) has found that *Pythium irregulare* can develop within host and nutrient solution toxin-like compounds which can act on sugar beet).

IV. Discussion and Conclusions

Some 5000 L.F. conductance readings were made during the above investigations. Provided due recognition was given to contamination of the inoculated host surfaces, partial desiccation of infected tissue, and conductance gradients within the tuber, L.F. conductance readings could invariably be used to determine the "injury" caused by pathogens. The differences between the effects of various "treatments" were reproducible and could be analysed statistically. Thus it was possible to follow quantitatively in time and space the injury caused by *Phytophthora* or *Pythium*. H.F. measurements were of use to show approximately what the L.F. conductance measurements would be if the tissues were dead. In general the probe method is better than the disc method, as it allows more rapid measurements and more precise location of injury.

According to the classical theory, the poor L.F. conductance of healthy tissue is due to the cell membrane being largely impermeable to ions. With increasing injury the membrane becomes more permeable and conductance increases. The similarity between the H.F. measurements on healthy and diseased (or even dead) tissues is due to the fact that the cell membranes (if present) do not impede the passage of H.F. currents (Höber 1945, p. 280). However, as these subjects are under investigation, it is proposed not to discuss them further. It must be realized (*a*) that the term "injury" is a general one and refers only to the effect of some injurious factor on the host tissue, resulting in this instance in an increase in L.F. conductance; (*b*) that many factors can cause injury. Therefore an increase in L.F. conductance does not give direct information on the underlying mechanism which causes the injury. We have already commented on the difference in host responses caused by *Phytophthora* and *Pythium*.

Nevertheless, L.F. conductance measurements proved valuable for measuring the velocity with which the host tissue responds to various strains of pathogens. Tables 3 and 4 show that injury develops more rapidly with a parabiotic combination of potato and *Phytophthora* than with a eusymbiotic combination. These results conform with previous ones obtained by other methods (Meyer 1939; Müller and Börger 1939; Müller, Meyer, and Klinkowski 1939). The results recorded in the two tables strongly support the claim that "reactive resistance" of potato to *Phytophthora infestans* actually depends on "supra-sensitivity" of the host plasm to the metabolic activities of the pathogen. They also support the claim that virulence of *P. infestans* is preconditioned by a certain degree of "tolerance" of the host tissue to the metabolites of the pathogen.

A further important result is that in a parabiotic combination increase in L.F. conductance was found in advance of the hyphal tips of the pathogen. From this we concluded that a parabiotic strain, as opposed to a eusymbiotic strain, affects not only the colonized area but also the adjacent area. At first sight an objection may be raised to this conclusion, in view of the similar curves in Figures 3(*a*) and 3(*b*). The objection would be that the "effect in advance" is only an apparent one, due to the slower and ultimately ceasing growth of the parabiotic strain, and the decrease of L.F. conductance is the same with increasing distance from the inoculated face. The objection implies (1) that changes in conductance occur with the same speed regardless of the growth rate of the different *Phytophthora* strains used, and (2) that there is no correlation between increase in conductance and virulence of the given *Phytophthora* strain as determined by depth of penetration. However, these two implications are contradicted by Tables 3 and 4 and by the observation that in the most resistant tubers an increase in L.F. conductance occurs over a distance of 3 mm or less. Therefore we conclude that the proposed objection is untenable, and that there is a real effect in advance.

Effects in advance are not new in plant pathology, a classic example being provided by the silver leaf disease of trees, caused by *Stereum purpureum*. Brooks and Brenchley (1931) showed that the "injury in advance" is caused by two chemical principles which are developed in the infected tissues of the stem or roots. From here the "toxins" travel through the vascular bundles to the leaves where they cause the silvering effect. Translocation of toxins from the infected tissue to other parts of the host has been shown in numerous wilt diseases (Gäumann 1951), and has been regarded as similar to the movement of tetanus toxin within a mammal, from the focus of infection to other parts of the body. But in all these cases the effect in advance is associated with susceptibility. Often, if the parasite subsequently colonizes the tissues "injured or killed in advance", the latter phenomenon has been regarded as essential for the establishment of the parasitic relationship. Such parasites are not parasites *sensu strictu* and have been termed "perthophytes", because they do not actually exist on the *living* substance of the host.

The *Phytophthora* disease of potato represents a parasitic relationship entirely distinct from that of diseases mentioned immediately above. Susceptibility to *Phytophthora* is preconditioned by the tolerance of the host tissue, as already mentioned, whereas resistance to *Phytophthora* results from premature death of the invaded tissue. On the basis of earlier experiments (Müller and Börger 1939; Müller *et al.* 1939) the checking of the growth of *Phytophthora*, observed in parabiotic combinations, can logicallybe explained in terms of an antibiotic principle (phytoalexine) which is activated in the colonized tissue in response to the infection. Simultaneous infection with *Phytophthora* strains interacting with the host tissue in a parabiotic and a eusymbiotic manner also resulted in both strains being checked. In addition it was shown that treatment with narcotics at concentrations which neither check the pathogen nor kill the host cells, caused a delay in the development of necrosis and correspondingly an increase in susceptibility (Behr 1949; Müller and Behr 1949). Accordingly if we admit that there is a defence mechanism *sensu strictu* which stops the pathogen's growth, we should expect a mechanism by which the host tissue adjacent to the invaded area is rendered inhospitable to the pathogen. If this were not so it is hard to understand why, in the otherwise unchanged tissue, the hyphal tips cease growing.

The effect in advance, as determined by L.F. conductance change, occurs over at least 4 mm, i.e. over a distance equivalent to at least 25 host cells. This is equivalent to at least 17 hr growth on the part of the parasite. Since in eusymbiotic combinations some 38 hr elapse before observable increase in conductance, the actual difference between a parabiotic and a eusymbiotic combination, expressed in terms of rate of interaction of host and parasite, is of the order of 55 hr at 20°C. This value indicates quite definitely the difference between the two types of interaction and emphasizes the dynamic nature of what we call resistance due to supra-sensitivity.

The chemical nature of the principle, which in parabiotic combinations affects conductance in advance, is at present unknown. Therefore the problem whether it plays an essential part in the defence reaction would at this stage be only a matter of vague speculation.

The type of injury caused by *Pythium* appears to be quite different from that caused by *Phytophthora*; accordingly, we have not used the term eusymbiotic combination to refer to *Pythium*-infected tissue, even though the host tissue is not injured in the region of the hyphal tips. We emphasize that the clinical response to *Pythium* differs largely from that to *Phytophthora*. Moreover, so far it has not been possible to obtain any evidence that the changes in the infected host cells are associated with any response checking the pathogen. *Pythium* appears to be a pathogen which kills the host (by means of a "vivotoxin"?) and lives readily on the substances released by the injured cells into the intercellular spaces. It appears to be intermediate in characteristics between an extreme perthophyte and an obligate parasite.

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VI. REFERENCES

BEHR, L. (1949).—*Phytopath. Z.* **15**: 407–46.

BLACK, W. (1952).—*Proc. Roy. Soc. Edin.* B **64**: 312.

BRANDENBURG, E. (1948).—*Z. PflKrankh.* **55**: 129–38.

BROOKS, F. T., and BRENCHLEY, G. H. (1931).—*New Phytol.* **30**: 128–35.

GÄUMANN, E. (1950).—"Principles of Plant Infection." (Transl. from German.) (Crosby Lockwood & Son: London.)

GÄUMANN, E. (1951).—"Pflanzliche Infectionslehre." 2nd Ed. (Birkhauser: Basel.)

GREENHAM, C. G. (1952).—*Nature* **170**: 535.

GREENHAM, C. G., and COLE, D. J. (1950).—*Aust. J. Agric. Res.* **1**: 103–17.

HÖBER, R. (1945).—"Physical Chemistry of Cells and Tissues." (Blakiston Co.: Philadelphia.)

LUYET, B. J. (1932).—*J. Gen. Physiol.* **15**: 283–7.

MEYER, G. (1939).—*Arb. biol. Abt.* (*Anst.-Reichsanst.*), *Berl.* **23**: 97–132.

MÜLLER, K. O. (1930).—*Angew. Botanik.* **12**: 299–324.

MÜLLER, K. O. (1953).—*J. Nat. Inst. Agric. Bot.* **6**: 346–60.

MÜLLER, K. O., and BEHR, L. (1949).—*Nature* **163**: 498–9.

MÜLLER, K. O., and BÖRGER, H. (1939).—*Arb. biol. Abt.* (*Anst.-Reichsanst.*), *Berl.* **23**: 189–231.

MÜLLER, K. O., MEYER, G., and KLINKOWSKI, M. (1939).—*Naturwissenschaften* **27**: 765–8.

OSTERHOUT, W. J. V. (1922).—"Injury, Recovery, and Death, in Relation to Conductivity and Permeability." (J. B. Lippincott: Philadelphia.)

STRUGGER, S. (1936).—*Protoplasma* **26**: 56–70.

RELATIONSHIPS OF LEGUME VIRUSES IN AUSTRALIA

I. STRAINS OF BEAN YELLOW MOSAIC VIRUS AND PEA MOSAIC VIRUS

By D. J. Goodchild*

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Summary

Two strains of bean yellow mosaic virus and two strains of pea mosaic virus are shown to be present in Australia, the physical properties and host ranges are reported, and the relationship between these and previously described strains is discussed.

In determining the presence of the viruses use is made of a series of nine differential hosts with which it is possible to differentiate the reported sap-transmissible virus diseases of peas and beans.

The economic significance of these diseases appears to be slight in crop plants, but could be of importance in pasture legumes, and, in particular, subterranean clover (*Trifolium subterraneum* L.).

Similarities in physical properties and symptom expression suggest a close relationship between bean yellow mosaic virus and pea mosaic virus.

I. Introduction

Sap-transmissible virus diseases of leguminous plants have been reported in Australia on previous occasions. Norris (1943) records the presence of pea mosaic in Western Australia, where species of lupins (*Lupinus* spp.) and subterranean clover (*Trifolium subterraneum* L.) were infected in the field. Aitken and Grieve (1943) record a mosaic virus disease of subterranean clover in Victoria, and Watson (1949) confirmed the presence of this virus, but suggested it to be a strain of bean yellow mosaic virus (B.Y.M.V.), of which more than one strain appeared to be present. Butler (1949, unpublished data) has recorded B.Y.M.V. in New South Wales, and Stubbs (1949, unpublished data) in Victoria, both on the basis of symptom expression on bean (*Phaseolus vulgaris* L.).

More recently Hutton and Peak (1954) described a B.Y.M.V. of subterranean clover which they considered, on a host-range basis, to be similar to that described by Pierce (1934).

For some years crops of peas and beans in New South Wales have shown a certain percentage of plants with mosaic symptoms, but no systematic study has been undertaken to determine the virus, or viruses, present. The economic aspects of the disease appeared to be slight, which is in accordance with observations made by Norris (1943) and Norris and Hutton (1943) on the effect of pea mosaic virus (P.M.V.) on the yield of seed from mechanically-inoculated pea plants in the field. Here, aphid populations were considered to be the limiting factor in the spread of this virus and also the fact that the variety Gem, or William Massey, a popular commercial variety, was immune.

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Table 1

SAP-TRANSMISSIBLE VIRUS DISEASES OF PEAS AND BEANS, SHOWING REACTIONS ON THE HOSTS NECESSARY FOR THEIR DIFFERENTIATION AND CLASSIFICATION

N = Necrosis; + = mottle; − = no reaction; LL = local lesions; CL = chlorotic local lesions; W = wilt

Virus	Authority	*Vicia faba* L. var. Windsor Long Pod	*Pisum sativum* L. var. Greenfeast†	*Phaseolus vulgaris* L. var. (*a*)‡(*b*)‡(*c*)‡	*Trifolium pratense* L.	*Trifolium incarnatum* L.	*Glycine max* (L.) Merrill	*Datura stramonium* L.	*Nicotiana glutinosa* L.	*Nicotiana tabacum* L.
Bean mosaic*	Weiss 1945	+	−	+$^{(c)}$	−	−		−	−	−
Bean southern mosaic*	Zaumeyer & Harter 1943	−	−	LL+$^{(a)}$	−	−	+	−	−	−
Bean yellow stipple	Zaumeyer & Thomas 1950	−	+$^{(p)}$	+$^{(b)}$	−	−	LL+			
Bean red node	Thomas & Zaumeyer 1950	N	N	N	+	+	+	+	−	+
Bean yellow mosaic*	Pierce 1934	+	+	+$^{(a)}$			+	−	−	−
(Pea mosaic)	Zaumeyer & Wade 1936	+	+	+	−		−	−	−	−
(Pisum 2)	Osborn 1937	+	+	+	+	+				
(Alsike clover mosaic)	Zaumeyer 1940	+	+	+$^{(a)}$	−	+	−			
(Severe pea)	Johnson & Jones 1937	+	+$^{(p)}$	+$^{(a)}$	+					−
(Pea mosaic 4)	Zaumeyer 1940	+	+	+	+	+	−	−	−	−
Bean yellow mosaic*	McWhorter *et al.* 1947	+	−	N	+	+	−			
(Subterranean clover mosaic)	Aitken & Grieve 1943	+	+	+$^{(a)}$	+	+	+			
(Type)	Grogan & Walker 1948	+	+	+$^{(b)}$	−	+	+	−	−	−

* These names taken from the "Virus Index" in *Rev. Appl. Mycol.* Vol. 24, No. 13, 1945.

† (*p*) = Perfection variety used in addition to Greenfeast.

‡ (*a*) = Corbett's Refugee; (*b*) = Blue Lake; (*c*) = Canadian Wonder.

TABLE 1 (*Continued*)

Virus	Authority	*Vicia faba* L. var. Windsor Long Pod	*Pisum sativum* L. var. Greenfeast†	*Phaseolus vulgaris* L. var. (*a*)‡(*b*)‡(*c*)‡	*Trifolium pratense* L.	*Trifolium incarnatum* L.	*Glycine max* (L.) Merrill	*Datura stramonium* L.	*Nicotiana glutinosa* L.	*Nicotiana tabacum* L.
(Pod-distorting)	Grogan & Walker 1948	−	+	$\mathrm{N}^{(b)}$	−	+	+	−	−	−
(Severe yellow)	Thomas & Zaumeyer 1953	+	+	$\mathrm{N}^{(b)}$	−	+	+	−	−	CL
(Necrotic lesion)	Zaumeyer & Fisher 1953	LL	−	$+^{(b)}$	−	−	+	−	−	LL
Bean yellow mosaic*	Fry 1953	+	+	$+^{(c)}$	+	−				
Bean yellow mosaic*	Chaudhuri 1950	+	+	+	−	+		−	−	−
Pea mosaic*	Doolittle & James 1925	+	+	−	+					
Pea mosaic*	Zaumeyer & Jones 1936	+	+	$-^{(a)}$	+		−	−	−	−
Pea mosaic*	Chamberlain 1936	+	+	−	+			−	−	−
(Pea 2A, 2B, 2C)	Stubbs 1937	+	+	$-^{(a)}$	−	+	−	−	−	−
(Pea 3)	Murphy & Pierce 1937	+	+	$-^{(a)}$	+	+	−	−	−	−
(Winconsin pea stunt) (Red clover vein mosaic)	Hagedorn & Hanson 1951	+	+	−	+	+	−			−

* These names taken from the "Virus Index" in *Rev. Appl. Mycol.* Vol. 24, No. 13, 1945.

† (*p*) = Perfection variety used in addition to Greenfeast.

‡ (*a*) = Corbett's Refugee; (*b*) = Blue Lake; (*c*) = Canadian Wonder.

TABLE 1 (*Continued*)

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Pea streak and stunt*	Chamberlain 1938	–	+	LL	+	LL	LL			
(Pea streak)	Zaumeyer 1938	+	+$^{(p)}$	–	+	+	+		–	–
Pea streak and stunt*	Ainsworth 1940	LL	+	+						
Pea streak and stunt*	Hagedorn & Walker 1949	–	+$^{(p)}$	–	+	–	–	–		+
(Pea wilt)	Johnson 1942	+N	W$^{(p)}$	+	+	+	–	–	–	–
Pea enation mosaic*	Osborn 1938	+	+	–	–	+	+		–	–
Alfalfa mosaic	Zaumeyer 1938	LL	+	LL$^{(a)}$	+	–	+	LL		+
Alfalfa mosaic	Krietlow & Price 1949	LL	+	LL	+	–	+	LL	+	+
(Alfalfa yellows)	Zaumeyer 1953	–	LL$^{(p)}$	LL$^{(b)}$	–	+	+	+	LL	LL
Broad bean mottle	Bawden *et al.* 1952	+	W	+$^{(c)}$	+	+	LL	–	–	–
Cucumber mosaic*	Whipple & Walker 1941	–	+	+			+	+	+	+
Pea mottle*§	Johnson 1942	+	+$^{(p)}$	+	+	+	–	–	–	–

* These names taken from the "Virus Index" in *Rev. Appl. Mycol.* Vol. 24, No. 13, 1945.

† (*p*) = Perfection variety used in addition to Greenfeast.

‡ (*a*) = Corbett's Refugee; (*b*) = Blue Lake; (*c*) = Canadian Wonder.

§ Pea mottle differs from viruses of the legume group by having many hosts outside the family Leguminosae.

The present investigation was commenced in 1953 with the initial object of determining the viruses prevalent in pea and bean crops, their host ranges, and possible potential. It was later extended to include pasture legumes and, in particular, subterranean clover.

In a survey of this type it seemed desirable to select an appropriate range of indicator plants whose reactions would enable the sap-transmissible virus diseases of peas and beans to be determined. An attempt was made to utilize the smallest host range possible which would differentiate these viruses. Weiss (1939) had proposed a scheme, but new viruses necessitated changes being made and, from a review of the literature, the differential hosts shown in Table 1 were selected.

Considerable confusion exists between different authors regarding the naming of P.M.V. and B.Y.M.V. and thus, for example, Osborn (1937), Pierce (1935), and Fry (1953) record P.M.V. as capable of infecting beans. With the system adopted in Table 1, these viruses are considered as B.Y.M.V. The table thus presents a convenient scheme for grouping, as well as differentiating, many of the sap-transmissible legume viruses, as the groupings become apparent when reactions on the hosts used are considered. Here, species differences have been utilized in establishing the groupings rather than differences in symptom expression or differences in varietal response within species. Most viruses affecting peas and beans are listed, together with the authority, and where a name differs from the group name this has been included in brackets.

II. Materials and Methods

(a) *General*

All investigations were made in the greenhouse where temperature variation was unfortunately large, varying from 11–33°C with the largest mean daily variation in winter of 14·1°C. In these studies consideration was given to previous investigations and an attempt to simulate the general methods of inoculation of previous investigators was made. Isolates obtained from field-infected plants were inoculated on to six *Phaseolus vulgaris*, var. Canadian Wonder, and six *Vicia faba* L., var. Windsor Long Pod, plants, but subsequent inoculations were made with sap expressed from infected broad beans. Commercial seed was used in all studies. Carborundum (180 mesh) was the abrasive, and plants were inoculated in the early stages of growth either with a pestle, or in the case of small-leaved plants, a ground-glass spatula. In all cases leaves were washed with water 1–2 min after inoculation.

Plants were grown in steam-sterilized soil in 4-in. pots, three plants per pot, and inoculated plants were retained for 6 weeks. In cases where symptoms were not visible, or were indistinct, an attempt was made to recover the virus inoculated, by sap inoculation from these plants to six bean and six broad bean plants of the above varieties.

The viruses used were isolated from the field in New South Wales with the exception of Phaseolus virus 2 Pierce (Hutton and Peak 1954), which was obtained from Dr. E. M. Hutton, Division of Plant Industry, C.S.I.R.O., Canberra, A.C.T. Subsequently, isolates of this virus were obtained from field-infected legumes.

Inoculum for physical tests was obtained from broad bean plants which had been showing symptoms for 1–2 weeks only. For each test, sap was obtained from a single plant and filtered through a cotton wool pad before treatment. When testing P.M.V. strains, broad beans were used as the indicator plants, but for strains of B.Y.M.V., beans were used as indicator plants. All plants were carefully selected for uniformity, and two expanded leaves of each broad bean and the two primary leaves of each bean inoculated respectively. In each test made, 24 plants were used as indicators, having regard to previous investigations, and the tests made as follows:

(i) *Dilution Tolerance.*—Sap from a single plant was used to prepare an appropriate dilution series in water.

Table 2

GROUPED REACTIONS OF ALL ISOLATES ON DIFFERENTIAL HOSTS

− = No symptoms; + = mottle symptoms

Species Inoculated	Bean Yellow Mosaic Virus		Pea Mosaic Virus	
	Strain 1	Strain 2	Strain 1	Strain 2
Vicia faba	+	+	+	+
Pisum sativum	+	+	+	+
Phaseolus vulgaris	+	+	−	−
Trifolium pratense	−	+	−	+
Trifolium incarnatum	+	+	+	+
Glycine max	+	+	−	−
Datura stramonium	−	−	−	−
Nicotiana glutinosa	−	−	−	−
Nicotiana tabacum	−	−	−	−

(ii) *Aging* in vitro.—In each time-interval test, 2 ml of sap were used and stored at 20±0·2°C for the required period.

(iii) *Thermal Inactivation.*—In each test, 2 ml of sap were placed in a thin-walled glass capsule and held in a water-bath for 10 min, which was agitated for the first minute. After 10 min the capsule was removed and immediately plunged into an ice-bath for rapid cooling, and the contents used to inoculate the indicator plants.

(*b*) *Species Trials*

These were carried out in the greenhouse using commercially available seed. In most cases 21 plants were inoculated with each isolate, three plants per 4-in. pot, and three uninoculated control plants retained. In all cases inoculum was obtained from broad bean plants showing symptoms for not longer than 3 weeks.

III. Results

(*a*) *Presence of Different Strains of B.Y.M.V. and P.M.V.*

The grouped results of reactions on the differential hosts, and varieties of those outlined in Table 1, are given in Table 2. Reference to Table 1 also shows, from the reactions outlined in Table 2, that strains of B.Y.M.V. and P.M.V. are responsible. All tests were duplicated and 12 plants were used in each test.

Descriptions of the symptoms caused by the B.Y.M.V. strains 1 and 2 and P.M.V. strains 1 and 2 on differential hosts are set out in Table 3. Physical tests, incorporating thermal inactivation, dilution tolerance, and aging *in vitro* of both strains of B.Y.M.V. and P.M.V., are given in Table 4, and host-range studies of these strains on leguminous hosts are shown in Table 5.

(*b*) *Symptom Expression of B.Y.M.V. Strain* 1 *on Various Hosts*

(i) *Beans.*—The varieties of bean tested can be grouped according to their symptom expression:

(1) *Top Necrosis and Severe Mottle.*—The second or third trifoliate leaves of beans in this group develop a severe yellow-green leaf mottle, which, in the case of the variety Epicure, is followed by veinal necrosis extending backward to the petiole (Plate 1, Fig. 2). Some necrosis of the growing point follows, but a percentage of recovery is noted and such plants have distorted leaves much reduced in size, and with a severe yellow-green leaf mottle. The varieties Epicure and Corbett's Refugee belong to this group.

(2) *Severe Mottle.*—Symptoms are similar to those already given for Canadian Wonder and the following varieties also react in a similar manner: Blue Lake, Bountiful, Brown Beauty, Burpee's Stringless Greenpod, Goward's Special, Kentucky Wonder, Michelite, Pinto, Refugee No. 5, Wellington Wonder, Windsor Long Pod, and the Wax variety of butterbean.

(3) *Mild Mottle.*—Sympton expression is characterized by a mild yellow-green leaf mottle and yellow-green leaf spots, but with no reduction in leaflet size (Plate 1, Fig. 3). Stringless Greenpod and Westralia varieties belong to this group and both tend to grow away from the virus under glasshouse conditions.

(ii) *Peas.*—Symptom expression is essentially similar for all varieties and, in general, is less severe than the symptom expression to the P.M.V. Vein yellowing is the first symptom, followed by either chlorotic spots or a general yellow-green leaf mottle with dark green vein-banded flecks in a few cases only.

(iii) *Clovers and Medics.*—Symptoms developed following inoculation by the *two* strains of B.Y.M.V. are essentially similar to those described for crimson clover (*Trifolium incarnatum* L.), and in almost all cases result in a reduction in leaf size, a severe yellow-green leaf mottle, and often distortion of the leaflets. Reactions of the varieties of subterranean clover are discussed later (see Section III (*e*)).

(iv) *Field Pea* (Pisum arvense *L.*), *Common Vetch* (Vicia sativa *L.*), *and Bokhara Clover* (Melilotus alba *Desr.*).—In each case a yellow-green leaf mottle with some dark green vein-banded flecks and chlorotic spots resulted.

(v) *New Zealand Blue Lupin* (Lupinus angustifolius *L.*).—With this species severe top necrosis without leaf symptom resulted 12–14 days after inoculation, but a small number of plants recovered. The new leaves of these plants had a brilliant yellow-green mottle, were reduced in size, and unfolded slowly or had recurved margins.

(vi) *Sweet Pea* (Lathyrus odoratus *L.*).—A mild mottle only resulted with this species and there was no reduction in leaf size or stunting of the plants.

TABLE 3

DESCRIPTION OF SYMPTOMS ON DIFFERENTIAL HOSTS

A. Bean Yellow Mosaic Virus Group

Host	Strain 1	Strain 2
Vicia faba	Diffuse mottle of 7th leaf 10–12 days after inoculation; 8th leaf with severe vein yellowing, and younger leaves with a yellow-green mottle, chlorotic spots, and dark green vein-banded flecks (Plate 1, Figs. 4, 5, and 6)	Similar to strain 1, not as marked, but mottle of yellow-green type with dark green vein-banded flecks
Pisum sativum	Vein yellowing followed by yellow-green mottle of leaves 12–15 days after inoculation. Younger leaves are slow to expand and have mild chlorotic spots and a mild mottle	Severe vein yellowing 10–14 days after inoculation, followed by a brilliant yellow-green leaf mottle and dark green vein-banded flecks. Leaves and stipules are slow to unfold
Phaseolus vulgaris	Faint chlorotic spots and vein clearing of second trifoliate leaf 6–10 days after inoculation. Younger leaves develop a severe yellow-green mottle, have extensive chlorotic areas, are relatively thick and brittle, and have recurved margins (Plate 1, Fig. 1)	Vein yellowing of the 2nd and 3rd trifoliate leaves 10–12 days after inoculation, followed by a yellow-green mottle or irregular chlorotic spots only in some cases (Plate 1, Fig. 4)
Trifolium pratense	No symptoms on 45 inoculated plants in three trials	Yellow-green leaf streaks on youngest leaves 10–12 days after inoculation. These become more pronounced in younger leaves and dark green vein-banded flecks may also appear (Plate 2, Fig. 11)
Trifolium incarnatum	Slight vein yellowing of youngest leaves 10–12 days after inoculation; subsequently leaves develop a brilliant yellow-green mottle with dark green vein-banded flecks (Plate 2, Fig. 10). Leaves reduced in size and plants stunted	Pronounced vein yellowing of youngest leaves 10–12 days after inoculation. A brilliant yellow-green leaf mottle follows and leaflets may be yellow-green with a few dark green vein-banded flecks. Leaves reduced in size, distorted, and plants stunted
Glycine max	Yellow-green mottle and irregular chlorotic spots but no reduction in leaf size	Similar to strain 1

TABLE 3 (*Continued*)

B. Pea Mosaic Virus Group

Host	Strain 1	Strain 2
Vicia faba	Vein yellowing of the 6th leaf followed by a general yellow-green leaf mottle which is not as severe as that of the B.Y.M.V. group	Vein yellowing and chlorotic spots on 6th leaf 10–12 days after inoculation, followed by a general yellow-green leaf mottle, more pronounced towards the leaf margins
Pisum sativum	Pronounced vein yellowing 12 days after inoculation followed by yellow-green streaks and dark green vein-banded flecks on youngest leaves	Vein yellowing followed by a brilliant yellow-green leaf mottle with dark green vein-banded flecks. Leaves and stipules slow to unfold
Trifolium pratense	No symptoms	Slight vein yellowing 10–12 days after inoculation followed by a mild mottle and no marked reduction in leaf size (Plate 2, Fig. 12)
Trifolium incarnatum	Pronounced vein yellowing followed by a brilliant yellow-green leaf mottle, dark green vein-banded flecks, and a reduction in leaf and plant size	Severe vein yellowing of youngest leaves 8–10 days after inoculation followed by a severe yellow-green leaf mottle, vein banded-flecks, leaf distortion, and marked stunting of infected plants

(*c*) *Symptom Expression of B.Y.M.V. Strain* 2 *on Various Hosts*

(i) *Beans.*—On beans symptoms were generally less severe than those recorded for strain 1, and the varieties tested showed general yellow-green leaf mottle symptoms with no reduction in leaf size or necrosis.

(ii) *Other Hosts.*—Symptom expression on the other hosts tested (Table 3) was similar to strain 1, and significant differences in symptoms could not be detected between the two strains except in reactions on subterranean clover (see Section III (*e*)).

(*d*) *Symptom Expression of P.M.V. Strains* 1 *and* 2 *on Various Hosts*

(i) *Peas.*—The mottles produced by viruses of this group are more severe than those produced by B.Y.M.V. in the greenhouse.

(ii) *Clovers and Medics.*—Symptom expression is similar to that described for B.Y.M.V. on crimson clover.

(iii) Cassia corymbosa *Lam.*—Following inoculation with P.M.V. strain 1, the young leaflets developed a yellow-green leaf mottle and dark green vein-banding, as described by Norris (1943).

(iv) *Field Pea and Common Vetch.*—Strain 2 of P.M.V. produced a distinct yellow-green leaf mottle in both species, but strain 1 could not be transmitted to the common vetch on three separate occasions involving 45 plants.

(v) *New Zealand Blue Lupin.*—Symptoms similar to those described by Norris (1943) and Chamberlain (1936) could not be produced on this host, nor was it a symptomless carrier for B.Y.M.V. as shown by Hagedorn and Walker (1949).

(vi) *Bokhara Clover.*—A very mild mottle resulted, together with the production of little-leaf symptoms, following inoculation with either strain of P.M.V. in the greenhouse, though this host is virtually a symptomless carrier.

TABLE 4

THERMAL INACTIVATION, DILUTION TOLERANCE, AND AGING IN VITRO OF BEAN YELLOW MOSAIC AND PEA MOSAIC VIRUS STRAINS

Results given as number infected/number inoculated

Test	Bean Yellow Mosaic Virus		Pea Mosaic Virus	
	Strain 1	Strain 2	Strain 1	Strain 2
Thermal inactivation				
50°C	19/23	—	—	—
55°C	17/23	9/23	18/24	3/24
58°C	0/24	6/23	17/24	1/24
60°C	0/24	3/24	13/24	1/24
62°C	—	0/24	14/24	0/24
64°C	0/24	0/24	0/24	0/24
Dilution tolerance				
1 in 10	14/24	—	15/24	13/24
1 in 100	4/24	13/24	12/24	3/24
1 in 1000	1/24	2/24	7/24	0/24
1 in 2000	0/24	1/24	2/24	0/24
1 in 5000	0/24	0/24	0/24	0/24
Aging *in vitro* (hr)				
24	8/24	19/24	2/20	5/24
36	9/24	—	—	2/24
48	6/24	18/24	2/24	6/24
60	2/24	13/24	7/24	1/24
72	1/24	9/24	0/24	0/24
84	1/24	—	—	—
96	0/24	0/24	—	—

(e) *Reactions of Subterranean Clover to B.Y.M.V. and P.M.V.*

The varieties shown in Table 5 were all tested in the greenhouse with both strains of B.Y.M.V. and P.M.V. Hutton and Peak (1954) report a lethal necrosis of the varieties Northam First Early, Pink Flowered, and Dwalganup when inoculated with Phaseolus virus 2 Pierce or strain 2 of B.Y.M.V. Plants of these varieties, of similar age, were inoculated with sap from broad beans infected with either strain of B.Y.M.V. and P.M.V. Only the B.Y.M.V. strain 2 of Hutton produced lethal necrosis on these varieties. The other strains of P.M.V. and B.Y.M.V. produced general yellow-green leaf mottles with some vein banding.

Variations in symptom expression between individual plants within varieties of subterranean clover made it impossible to detect differences in symptom expression produced by the different strains. In general, the varieties Clare and Yarloop responded with the most brilliant yellow-green leaf mottles and the greatest reduction in leaf size and distortion of leaflets.

A severe field infection of a varietal trial of subterranean clover was observed in the spring of 1954. Four isolates, from among the 21 varieties, indicated that P.M.V. strain 2 was responsible. In the field the varieties Yarloop and Clare showed severest infection, having a marked reduction in leaf size and consequent stunting of plants.

A severe field infection was also observed in a subterranean clover field trial at Dickson Experiment Farm, Canberra, A.C.T. B.Y.M.V. strain 2 was isolated from three varieties, and from red clover bordering the field. A notable feature of the symptom expression of some introduced varieties was a lack of distinct mottle symptoms but a definite reduction in leaf size, resulting in a "little-leaf" condition of infected plants, usually accompanied by a marked reddening of the petiole and lamina.

(*f*) *Field Isolations of P.M.V. and B.Y.M.V. Strains* 1 *and* 2

Viruses of both groups were isolated from 17 different areas. On five occasions B.Y.M.V. strain 1 was isolated, and B.Y.M.V. strain 2 was isolated on four occasions. P.M.V. strain 1 was isolated on two occasions only, but P.M.V. strain 2 was isolated on six occasions. From the limited nature of such a survey it is not possible to map accurately the distribution, nor determine the hosts most usually affected and the percentage of infection.

Symptom expression on field-infected hosts usually differed from that observed in the greenhouse. Except in the case of subterranean clover already mentioned, plants in the field were less severely affected by the viruses studied.

B.Y.M.V. strain 1 was isolated from field-infected beans, variety Canadian Wonder, having typical yellow-green leaf symptoms, but the reduction in leaf size was not severe so that plants were not unduly stunted. Peas affected by this virus also, although with clearly-defined mottle symptoms, were not markedly stunted. Symptom expression on a field-infected gladiolus (*Gladiolus cuspidatus* Jacq.) was characterized by a diffuse yellow-green leaf mottle and irregular yellow-green patches.

P.M.V. strain 1 was obtained from pea, variety Greenfeast, showing a general yellow-green leaf mottle and some reduction in leaf size, but with no distortion of leaflets or stipules. Field-infected broad beans, however, show only diffuse chlorotic spots. P.M.V. strain 2 was isolated from pea, variety Greenfeast, having a pronounced yellow-green leaf mottle with some vein banding and dark green vein-banded flecks towards the margins, but there was little reduction in leaf size. Broad bean, field-infected with this strain, showed marked vein banding.

(*g*) *Differences in Severity of Isolates of B.Y.M.V. Strain* 1

An isolate of this strain was obtained from pea, variety Greenfeast, infected in the field, which produced a mild mottle only in bean, variety Canadian Wonder.

Expanded leaves developed distinct chlorotic spots 4–6 weeks after inoculation and leaves developed later were reduced in size and had a severe yellow-green mottle. When transfers were made from these beans to others, symptoms appeared after 12–14 days. This isolate also produced more severe symptoms in pea varieties than did B.Y.M.V. strain 1 already described. This is shown in Plate 2, Figures 7 and 8,

Table 5

REACTIONS OF STRAINS OF BEAN YELLOW MOSAIC VIRUS AND PEA MOSAIC VIRUS ON LEGUMINOUS HOSTS

+ = Mottle symptoms; N = necrotic symptoms; — = no symptoms

Species	Variety	Bean Yellow Mosaic Virus		Pea Mosaic Virus	
		Strain 1	Strain 2	Strain 1	Strain 2
Cassia corymbosa Lam.	—	—	—	+	—
Lathyrus odoratus L.	—	+			
Lupinus angustifolius L.	—	+	+	—	—
Medicago denticulata Willd.	—	+	+	—	+
M. laciniata (L.) Mill.	—	+	+	+	+
M. sativa L.	—	—	—	—	—
M. tribuloides Desr.	—	+	+	—	—
Melilotus alba Desr.	—	+	+	+	+
Phaseolus lunatus L.	—	—	—	—	—
Phaseolus vulgaris L.	Blue Lake	+	+	—	—
	Bountiful	+			
	Brown Beauty	+	+	—	—
	Burpee's Stringless Greenpod	+			
	Corbett's Refugee	+	+	—	—
	Epicure	+	+	—	
	Goward's Special	+			—
	Gt. Northern U.1 No. 1	—	—		
	Kentucky Wonder	+	+	—	—
	Michelite	+			
	Pinto	+		—	—
	Refugee No. 5	+		—	—
	Startler Wax	+			
	Stringless Greenpod	+	+	—	—
	Wellington Wonder	+			
	Westralia	+	+	—	—
	Windsor Long Pod	+	+	—	—
Pisum arvense L.	Dun, Grey	+	+	+	+
Pisum sativum L.	Wm. Massey (Gem)	—	—	—	—
	Canner's Perfection 53	—		—	—
	Canner's Perfection 75	+		+	+
	Telephone	+	+	+	+
	Witham Wonder	—	—	—	—
	Yorkshire Hero	+	+	+	+

TABLE 5 (*Continued*)

Species	Variety	Bean Yellow Mosaic Virus		Pea Mosaic Virus	
		Strain 1	Strain 2	Strain 1	Strain 2
Trifolium repens L.	—	—	—	—	—
Trifolium subterraneum L.	Bacchus Marsh	+	+	+	+
	Clare	+	+	+	+
	Dwalganup	+	+N	+	+
	Macarthur			+	+
	Mt. Barker	+	+	+	+
	Mulwala	+	+	+	+
	Northam First Early	+	+N	+	+
	Pink Flowered	+	+N	+	+
	Ruakura			+	+
	Tallarook	+	+	+	+
	Wenigup	+	+	+	+
	Yarloop	+	+	+	+
Vicia sativa L.	—	+	+	—	+
Vigna sinensis (L.) Endl. ex Nassk.	—	—	—	—	—

where differences in the symptom expression are shown on the pea variety Telephone. There was also a difference in thermal inactivation and dilution tolerance:

Physical Test	B.Y.M.V. Strain 1	
	Severe Isolate	Mild Isolate
Thermal inactivation	55–58°C	60–64°C
Dilution tolerance	1 in 1000–1 in 2000	1 in 2000–1 in 5000

The host ranges of both isolates were similar with respect to 46 hosts.

IV. DISCUSSION

Difficulties in deciding the criteria to be adopted when classifying and distinguishing viruses of the legume group presents a major problem. This seems partially due to the failure of earlier investigators to appreciate the very wide host range capacity of viruses of the group. Thus descriptions appeared of bean mosaics, pea mosaics, and sweet clover mosaic viruses as these viruses were described on new host plants. Later workers tended to describe, in detail, differences between strains of these viruses, utilizing as criteria differences in symptom expression on selected varieties of peas and beans. While in some cases these may have been justified from differences in host ranges, in others the differences were not of sufficient magnitude to warrant naming of new strains. Further confusion has been created between the pea and bean mosaic viruses by descriptions of pea mosaics which attack beans. Thus Fry (1953) describes a B.Y.M.V. which he considers similar to the P.M.V. of Chamberlain (1936) but which now attacks beans.

In Table 1 an attempt has been made to provide a means of grouping the sap-transmissible virus diseases of peas and beans by the utilization of a series of differential host plants. This is not intended as a method of classification, but it is suggested that such groupings may provide a basis for the development of a system of classification for viruses of this group. Use has been made of the fact that species differences in host range would seem *a priori* to be a better criterion for differentiation than varietal differences within species or symptom expression within varieties, which can vary a great deal with environmental factors. Thomas and Zaumeyer (1953) have introduced a similar scheme but with a larger number of differential hosts for differentiating viruses of the B.Y.M.V. group, and recognize six strains of the virus.

Physical tests have been used as criteria when describing viruses of the legume group, and particularly when differentiating strains of B.Y.M.V. and P.M.V. The success of such tests depends upon the concentration of virus in the treated inoculum, the technique of inoculating indicator plants, and the number of plants inoculated. Only small differences between virus strains are reported in some cases and this is particularly evident with thermal inactivation values. It is doubtful if 2°C differences in thermal inactivation can be considered to be real when the number of variable factors associated with the technique are considered.

Zaumeyer (1940) has tabulated results of physical tests on 14 legume viruses and it is readily seen that overlapping in the physical properties occurs, particularly between strains of B.Y.M.V. and P.M.V. It seems, then, that these tests have doubtful value even as an adjunct to host-range studies, and that it is essential to derive a suitable set of indicator hosts similar to that proposed in Table 1.

It is at once apparent, however, that different workers have not been consistent in the use of similar varieties of the differential hosts, and particularly is this the case for peas and beans. It is thus impossible to fit all described legume viruses to one scheme. A large number have, however, utilized Corbett's Refugee as the bean variety, this being immune to B.Y.M.V. With pea varieties, Greenfeast types have been selected as a rule, together with the resistant Perfection type as a check.

Major differences in host range and host reaction are apparent between several of the viruses, viz. bean mosaic, bean southern mosaic, bean yellow stipple, bean red node, alfalfa mosaic, and broad bean mottle virus. However, greatest confusion exists between the P.M.V. and B.Y.M.V. groups, which differ significantly from those listed above but which do not differ greatly from each other in host range or host reaction. The basic difference lies in the inability of P.M.V. to infect beans. Within each group several strains can be recognized, chiefly by the different reactions on red and crimson clovers, and on soybeans. The pea mosaic viruses reported by Murphy and Pierce (1937) and Hagedorn and Hanson (1951) would appear to be similar from the reactions on the nine hosts in Table 1 and they differ chiefly in the severity of reaction on peas.

The B.Y.M.V. group presents a more complex problem, but it is possible to differentiate most of the components; however, in some cases, insufficient information is available for adequate comparison. Those viruses whose host ranges correspond over the nine indicator hosts are found to be closely related and differentiated only by differences in bean varietal reactions. Thus the host range described by Johnson and Jones (1937) for severe pea mosaic (which must be considered a strain of B.Y.M.V. on host reactions) is similar to that described by Zaumeyer (1940) for pea mosaic virus 4 (also a strain of B.Y.M.V.). The difference lies in the latter's inability to infect Corbett's Refugee, Wisconsin Refugee, or Great Northern U.1 No. 1 beans, and the former's ability to infect Perfection peas.

The B.Y.M.V. strain 1 reported in this investigation has a host range similar to that recorded by Grogan and Walker (1948) for their "type strain". Symptom expression and host range on the bean varieties was similar to that described, and included the inability to infect the Great Northern U.1 No. 1 bean variety. Similar symptoms were also observed on field pea, crimson clover, common vetch, broad bean, and soybean and no symptoms on lima bean and white clover; burr medic, however, was susceptible. The physical properties differ slightly, as Grogan and Walker record a thermal inactivation of 58–60°C compared with 55–58°C, but the aging *in vitro* differed markedly, being 84–96 hr compared with 24–48 hr.

Evidence has been presented to show the existence of a less severe isolate of this strain, which is similar in all respects except in the severity of symptoms and a slight difference in physical properties. Symptom expression on beans is similar to that recorded by Zaumeyer and Wade (1936) for pea virus 2 (a strain of B.Y.M.V.), but host range and physical properties are not similar and the isolate cannot be considered as being similar to this strain of B.Y.M.V. These results do show, however, the variation occurring between isolates of apparently distinct strains of B.Y.M.V. and the probable existence of a range of strains within and between existing strains of these viruses, possibly due to a process of continuous variation in the virus.

The second strain of B.Y.M.V. has been described by Hutton and Peak (1954) and, as suggested by them, is similar to that described by Pierce (1934). The subterranean clover mosaic of Aitken and Grieve (1943) also appears to belong to this group. The physical properties differ from those reported by Pierce. Aging *in vitro* is 72–96 hr compared with 28–32 hr, dilution tolerance is 1 in 2000–1 in 5000 compared with 1 in 800–1 in 1000, and the thermal inactivation is 60–62°C compared with 56–58°C. This second strain is somewhat similar in host range to strain 1 but differs in the ability to infect red clover and to cause necrosis in early varieties of subterranean clover.

Strain 1 of P.M.V. is similar in host range to pea viruses 2A, 2B, and 2C described by Stubbs (1937). Differences are apparent in the physical properties as Stubbs' strains have a dilution tolerance of 1 in 1000–1 in 1500 compared with 1 in 2000–1 in 5000, and an aging *in vitro* of 24 hr compared with 60–72 hr for the virus reported here. This strain is also similar in most respects to the P.M.V. described by Norris (1943) in Western Australia and has in common the ability to infect the shrub *Cassia corymbosa*, which is not shared by the other viruses studied.

The remaining strain of P.M.V. is similar in host range to pea virus 3 of Murphy and Pierce (1937) and the physical properties also bear a close relationship. Murphy and Pierce record thermal inactivation between 58 and 60°C compared with 60–62°C, and aging *in vitro* of 2–3 days compared with $2\frac{1}{2}$–3 days reported here. The only difference in host range was an inability to cause necrosis of *Lupinus angustifolius* although several tests were carried out. This strain is also similar to that described by Chamberlain (1936) in New Zealand and Zaumeyer and Wade (1936) in the United States of America.

Similarity in symptom expression for the different viruses on their common differential hosts is evident from the symptom descriptions given, and the viruses could not be satisfactorily separated by this means. Clonal and environmental variations are probably largely responsible, and in an experiment with broad beans inoculated and grown under controlled conditions of light and temperature, variation in symptom expressions among plants was so great that no differences between the viruses could be detected.

The wide host ranges of the viruses reported indicates the ability of species of legumes present in natural and established pastures in Australia to provide an adequate reservoir of virus. This fact could be of importance in the establishment and maintenance of subterranean clover pastures as all varieties of this species tested are particularly susceptible to both B.Y.M.V. and P.M.V. The relatively low incidence of virus in pastures and in commercial bean crops may be a reflection upon the efficacy of insect transmission, but the potential, nevertheless, is present. With peas, however, the wide use of the variety Gem or William Massey for market consumption, and the use of Canner's Perfection 75 in the canning industry has virtually eliminated virus as a disease of this crop.

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VI. References

Ainsworth, G. C. (1940).—The identification of certain viruses found infecting leguminous plants in Great Britain. *Ann. Appl. Biol.* **27**: 218.

Aitken, Y., and Grieve, B. J. (1943).—A mosaic disease of sub. clover. *J. Aust. Inst. Agric. Sci.* **9**: 81–2.

Bawden, F. C., Chaudhuri, R. P., and Kassanis, B. (1952).—Some properties of broad bean mottle virus. *Ann. Appl. Biol.* **34**: 774–84.

Chamberlain, E. E. (1936).—Pea-mosaic. Host range and methods of transmission. *N.Z. J. Sci. Tech.* **18**: 544–56.

Chamberlain, E. E. (1938).—Pea-streak (*Pisum Virus* 3). *N.Z. J. Sci. Tech.* A **20**: 365–81.

Chaudhuri, R. P. (1950).—Studies on two aphid-transmitted viruses of leguminous crops. *Ann. Appl. Biol.* **37**: 342–54.

Doolittle, S. P., and Jones, F. R. (1925).—The mosaic disease in garden pea and other legumes. *Phytopathology* **15**: 763–71.

Fry, P. R. (1953).—Two virus diseases of gladiolus. *N.Z. J. Sci. Tech.* A **34**: 460–4.

Grogan, R. G., and Walker, J. C. (1948).—A pod-distorting strain of the yellow mosaic virus of bean. *J. Agric. Res.* **77**: 301–14.

Hagedorn, D. J., and Hanson, E. W. (1951).—A comparative study of the viruses causing Wisconsin pea stunt and red clover vein mosaic. *Phytopathology* **41**: 813–19.

Hagedorn, D. J., and Walker, J. C. (1949).—Wisconsin pea streak. *Phytopathology* **39**: 837–47.

HUTTON, E. M., and PEAK, J. W. (1954).—Varietal reactions of *Trifolium subterraneum* L. to Phaseolus virus 2 Pierce. *Aust. J. Agric. Res.* **5**: 598–607.

JOHNSON, F. (1942).—The complex nature of white clover mosaic. *Phytopathology* **32**: 103–16.

JOHNSON, F., and JONES, L. K. (1937).—Two mosaic diseases of peas in Washington. *J. Agric. Res.* **54**: 629–38.

KRIETLOW, K. W., and PRICE, W. C. (1949).—A new virus disease of Ladino clover. *Phytopathology* **39**: 517–28.

McWHORTER, F. P., BOYLE, L., and DANA, B. F. (1947).—Production of yellow bean mosaic in beans by virus from mottled gladioli. *Science* **105**: 177–8.

MURPHY, D. M., and PIERCE, W. H. (1937).—Common mosaic of the garden pea *Pisum sativum*. *Phytopathology* **27**: 710–21.

NORRIS, D. O. (1943).—Pea mosaic on *Lupinus varius* L. and other species, in Western Australia. Bull. Coun. Sci. Industr. Res. Aust. No. 170.

NORRIS, D. O., and HUTTON, E. M. (1943).—Pea mosaic, with special reference to its effects on yield of seed. *J. Coun. Sci. Industr. Res. Aust.* **16**: 149–54.

OSBORN, H. T. (1937).—Vein mosaic of red clover. *Phytopathology* **27**: 1050–8.

OSBORN, H. T. (1938).—Studies on pea virus 1. *Phytopathology* **28**: 923–34.

PIERCE, W. H. (1934).—Viruses of the bean. *Phytopathology* **24**: 87–115.

PIERCE, W. H. (1935).—The identification of certain viruses affecting leguminous plants. *J. Agric. Res.* **51**: 1017–39.

STUBBS, M. W. (1937).—Certain viruses of the garden pea. *Phytopathology* **27**: 242–66.

THOMAS, H. R., and ZAUMEYER, W. J. (1950).—Red node, a virus disease of bean. *Phytopathology* **40**: 832–59.

THOMAS, H. R., and ZAUMEYER, W. J. (1953).—A strain of yellow bean mosaic virus producing local lesions on tobacco. *Phytopathology* **43**: 11–15.

WATSON, MARION A. (1949).—Some notes on plant virus diseases in South Australia. *J. Aust. Inst. Agric. Sci.* **15**: 86.

WEISS, F. (1939).—Key to typical viruses of leguminous crops. *Plant Dis. Reptr.* **23**: 352.

WEISS, F. (1945).—Viruses described primarily on leguminous vegetable and forage crops. *Plant Dis. Reptr.* Suppl. No. 154.

WHIPPLE, O. C., and WALKER, J. C. (1941).—Strains of cucumber mosaic virus pathogenic on bean and pea. *J. Agric. Res.* **62**: 27–60.

ZAUMEYER, W. J. (1938).—A streak disease of peas and its relation to several strains of alfalfa mosaic virus. *J. Agric. Res.* **56**: 747–72.

ZAUMEYER, W. J. (1940).—Three previously undescribed mosaic diseases of pea. *J. Agric. Res.* **60**: 433–52.

ZAUMEYER, W. J. (1953).—Alfalfa yellow mosaic virus systematically infectious to beans. *Phytopathology* **43**: 38–44.

ZAUMEYER, W. J., and FISHER, H. H. (1953).—A new necrotic lesion-producing strain of yellow bean mosaic. *Phytopathology* **43**: 45–9.

ZAUMEYER, W. J., and HARTER, L. L. (1943).—Two new virus diseases of beans. *J. Agric. Res.* **67**: 305–28.

ZAUMEYER, W. J., and THOMAS, H. R. (1950).—Yellow stipple, a virus disease of bean. *Phytopathology* **40**: 847–59.

ZAUMEYER, W. J., and WADE, B. L. (1936).—Pea mosaic and its relation to other legume mosaic viruses. *J. Agric. Res.* **53**: 161–85.

EXPLANATION OF PLATES 1 AND 2

Symptoms developed on various hosts

PLATE 1

Fig. 1.—Leaves of bean, var. Canadian Wonder, showing a yellow-green leaf mottle, vein clearing, and a marked reduction in size following inoculation with B.Y.M.V. strain 1.

Fig. 2.—Ventral surface of a leaf of bean, var. Epicure, showing a systemic necrosis of the veins which extends to the petiole, following inoculation with B.Y.M.V. strain 1.

Fig. 3.—Mild yellow-green chlorotic spots are developed on bean, var. Stringless Greenpod, following inoculation with B.Y.M.V. strain 1.

Fig. 4.—Leaf of bean, var. Canadian Wonder, showing vein yellowing and irregular chlorotic spots which appear on young leaves approx. 2 weeks after inoculation with B.Y.M.V. strain 2.

Fig. 5.—Mottle developed on the 7th leaf of broad bean, var. Windsor Long Pod, following inoculation with B.Y.M.V. strain 1. This is characterized by large yellow-green areas and is followed by the general mottle symptoms shown in Plate 1, Figure 6.

Fig. 6.—Leaves of broad bean, var. Windsor Long Pod, showing the general yellow-green leaf mottle with irregular chlorotic areas developed 2–3 weeks after inoculation with B.Y.M.V. or P.M.V. strains.

PLATE 2

Fig. 7.—Pea, var. Telephone, showing mild chlorotic mottle symptoms, with distinct chlorotic spots, developed on leaves following inoculation with the mild isolate of B.Y.M.V. strain 1.

Fig. 8.—Pea, var. Telephone, showing distinct chlorotic spots, dark green vein-banded flecks, and yellow-green leaf streaks; symptoms developed after inoculation with the severe isolate of B.Y.M.V. strain 1.

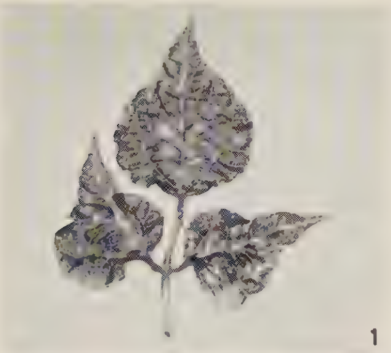
Fig. 9.—Pea, var. Telephone, with general yellow-green leaf mottle and vein-banding of leaves developed after inoculation with P.M.V. Compare with Plate 1, Figure 5, and Plate 2, Figures 7 and 8.

Fig. 10.—A range of mottle symptoms developed on crimson clover (*Trifolium incarnatum*) after inoculation with B.Y.M.V. strain 1. The leaf on the left shows the most severe symptoms with a general yellowing of the leaflets and pronounced dark green vein-banded flecks. The other leaves show chlorotic spots and vein yellowing and all are reduced in size.

Fig. 11.—Red clover (*Trifolium incarnatum*) showing yellow-green leaf streaks and vein-banded flecks developed following inoculation with B.Y.M.V. strain 2.

Fig. 12.—Red clover (*Trifolium incarnatum*) with mild yellow-green leaf mottle, and some vein-banded flecks, which develop after inoculation with P.M.V. strain 2.

RELATIONSHIPS OF LEGUME VIRUSES IN AUSTRALIA. I



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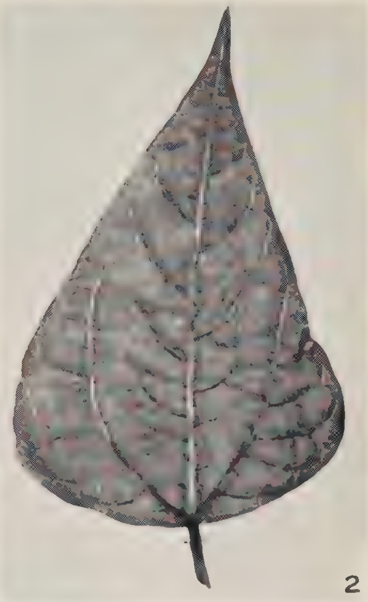

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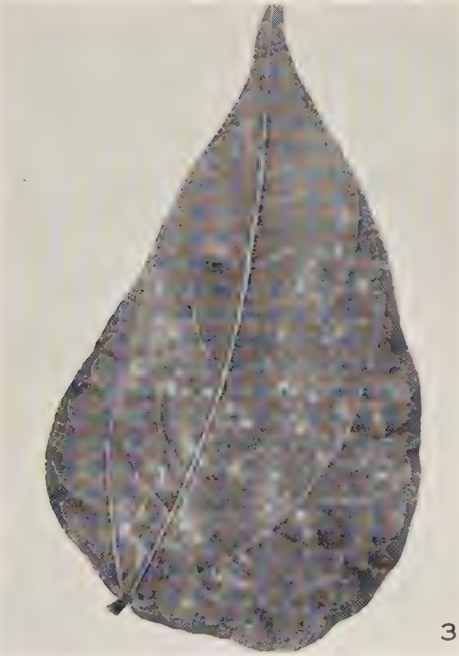

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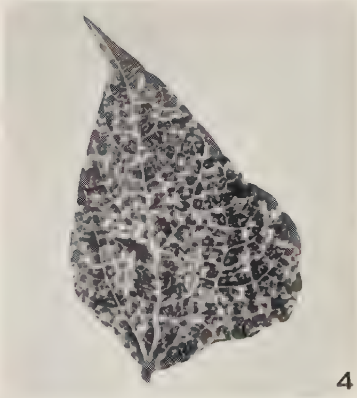

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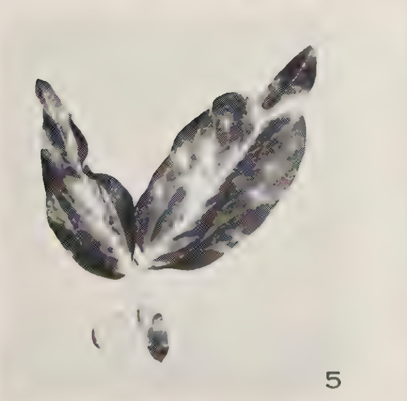

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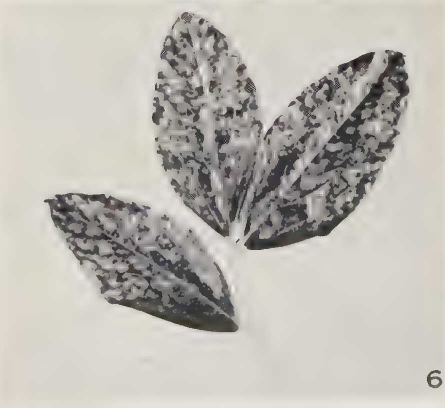

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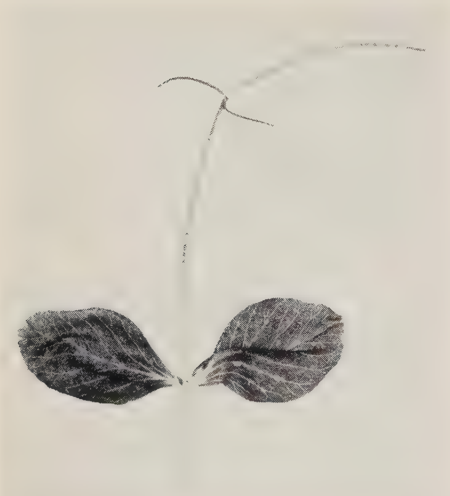
RELATIONSHIPS OF LEGUME VIRUSES IN AUSTRALIA. I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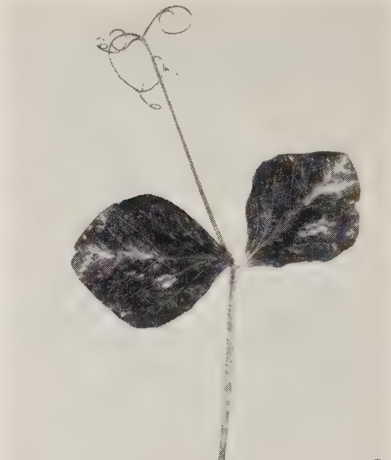
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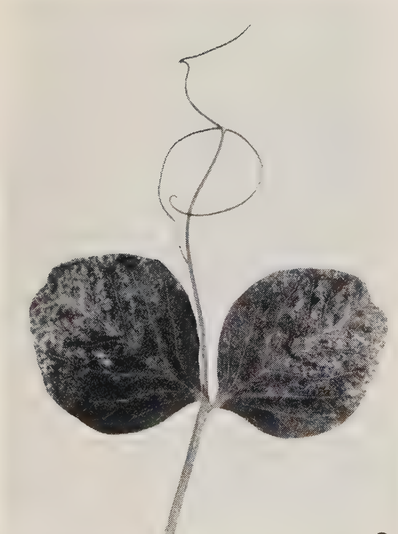
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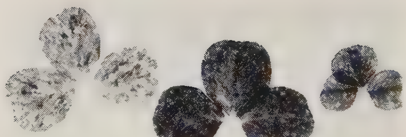
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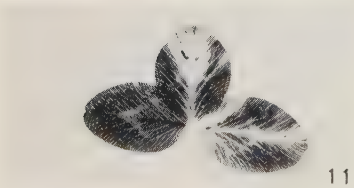
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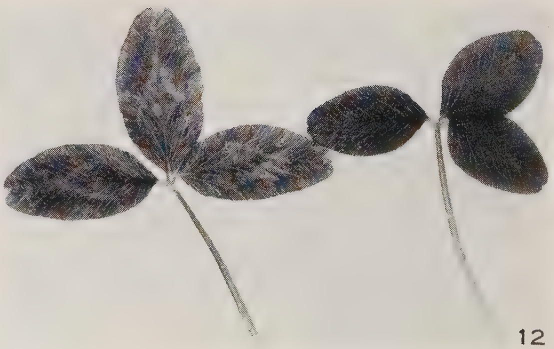
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RELATIONSHIPS OF LEGUME VIRUSES IN AUSTRALIA

II. SEROLOGICAL RELATIONSHIPS OF BEAN YELLOW MOSAIC VIRUS AND PEA MOSAIC VIRUS

By D. J. Goodchild*

[*Manuscript received August* 29, 1955]

Summary

Antisera were prepared to one strain of bean yellow mosaic virus (B.Y.M.V.) and two strains of pea mosaic virus (P.M.V. strain 1 and P.M.V. strain 2). By utilizing the agglutination reaction, the serological activity of both strains of P.M.V. is demonstrated for the first time. The results of cross-agglutination experiments show the strains of P.M.V. and B.Y.M.V. to be serologically related. Cross-protection tests support the hypothesis that these are strains of one and the same virus.

Serological relationships within the legume virus group are discussed.

I. Introduction

The demonstration of serological activity for bean yellow mosaic virus (B.Y.M.V.) and pea mosaic virus (P.M.V.) has presented difficulties. Bawden (1948) discusses the probable reasons for this, and considers low concentration of virus within the host to be the most serious limiting factor. Increasing attention is now being directed towards techniques of antiserum production and the use of suitable adjuvants may well play an important role in plant virus serology. New techniques associated with demonstrating antigen-antibody reactions are being developed (van Slogteren 1955) and this should also help to increase the number of known serologically active plant viruses.

It has been considered desirable to purify partially the plant virus antigen before inoculating the animal to be used in antiserum production. Beemster and van der Want (1951) used massive doses of dialysed infected sap, injected intravenously, as antigen for antibody production. They were able to demonstrate serological activity for bean mosaic virus and B.Y.M.V. and laid the foundations for antiserum preparation to many viruses considered to be serologically inactive. In achieving this, use was made of the agglutination reaction for demonstrating antigen-antibody reactions, first described for plant viruses by Chester (1937). Precipitin reactions could not be demonstrated, but sufficiently reproducible results were obtained by the agglutination technique for it to be utilized in determining serological relationships within the legume virus group. Beemster and van der Want showed bean mosaic virus and B.Y.M.V. to be serologically similar, and the work reported here shows the relationships between the strains of B.Y.M.V. and P.M.V. reported by Goodchild (1956).

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II. Materials and Methods

Antiserum was prepared to strain 1 of B.Y.M.V. (Grogan and Walker 1948) and to the two strains of P.M.V. (Murphy and Pierce 1937; Stubbs 1937) reported in a previous paper (Goodchild 1956). Antigen was obtained from plants of *Vicia faba* L., var. Windsor Long Pod, which had been showing symptoms for at least 2 weeks but not longer than 4 weeks. Sufficient antigen for one inoculation was usually obtained from a single plant. Antigen was prepared by crushing plants in a mortar with a pestle, expressing the sap, and filtering first through a cotton wool pad and then through a Whatman No. 541 filter paper, using a Buchner funnel.

The filtered sap, which was dialysed against running tap water for 3 hr in a "Cellophane" bag, constituted the antigen. Rabbits were used to produce antiserum and each received 12 intravenous injections of 4 ml, four each week for 3 weeks with a 3-day interval at weekends. In the case of P.M.V. strain 1, however, sufficient antigen for 10 injections only was available. Several millilitres of normal serum were obtained from the marginal ear vein of each rabbit before injections commenced, and each serum sample was retained separately.

Eight days after the final injection blood was taken from the jugular vein of each rabbit and allowed to clot at 37°C for 1 hr and then at 3°C for a further 2 hr. Antiserum was separated by decantation and clear-centrifuged (10 min at 3000 r.p.m.). Normal serum was separated in a similar manner and all sera were stored in small ampules at —20°C until required. This procedure was adopted as Beemster and van der Want (1951) considered that the antiserum lost activity if retained under normal storage conditions. Two rabbits died, but anaphylaxis was not the cause in either case as shown by post-mortem examination.

The micro-technique developed by van Slogteren and others was used by Beemster and van der Want to demonstrate agglutination reactions, but due to technical difficulties it was decided to adapt the agglutination tube technique for these reactions. Dreyer-type tubes, with a capacity of approximately 1 ml, were used and in each case 0·5 ml of antigen was added to 0·5 ml of antiserum. All dilutions of antigen and antiserum were made with 0·85 per cent. saline, and for each antigen-antiserum test, controls of healthy, dialysed sap-antiserum, antigen-normal serum, and healthy, dialysed sap-normal serum were run. All tubes were incubated in racks in a water-bath.

III. Results

(a) *Agglutination Technique*

(i) *Optimum Agglutinating Temperature*.—As the antigen in all agglutination tests consisted of filtered infective sap, containing a large proportion of cell components, this would be expected to precipitate spontaneously, particularly at high temperatures. At low temperatures, however, the antigen-antibody reaction would proceed slowly so that effective agglutination may not be possible. Therefore, the temperature desired was one at which spontaneous precipitation did not take place at such a rapid rate that the antigen-antibody reaction could not be separated from it. Normal bacterial agglutinations take from 1–3 hr, so that 3 hr was considered to be the minimum time before which spontaneous precipitation should occur.

An arbitrary saturation (see Section III (*a*) (ii)) of 1 : 30 with normal serum was made and this then diluted 1 : 1, 1 : 2, 1 : 4, and 1 : 8. Healthy, dialysed sap was diluted 1 : 2 and 1 : 4 and added to separate series of tubes which were incubated at 30, 32, 35, 37, and 52°C. Only slight precipitation after 3 hr resulted at 30, 32, and 35°C, but bulky precipitates were evident 1 hr after incubation at 37 and 52°C, so that 35°C was used for all subsequent reactions.

(ii) *Saturation of Antiserum.*—Antibodies are developed to plant proteins, as well as virus protein, when crude infective sap is used as an antigen. These are removed by saturating the antiserum with crude, uninfected sap and centrifuging, when the clear supernatant antiserum is used for agglutination or precipitin reactions. For each antiserum the optimum saturation point was determined by saturating the antisera at 25°C for 3 hr and attempting agglutinations with healthy antigen. Saturation was achieved with healthy, broad bean sap diluted 1 : 1 with saline.

Table 1

DETERMINATION OF ANTISERA TITRE

+ = Agglutination; ± = slight agglutination; — = no agglutination

Antigen	Homologous Saturated Antiserum Dilutions						
	1:1	1:2	1:4	1:8	1:16	1:32	1:64
Bean yellow mosaic virus	+	+	+	+	±	—	—
Pea mosaic virus strain 1	+	+	+	+	+	±	—
Pea mosaic virus strain 2	+	+	+	+	+	±	—

For B.Y.M.V. the optimum saturation ratio was found to be 1 : 24, while for P.M.V. strain 1 this was 1 : 18 and 1 : 22 for P.M.V. strain 2. At saturations below the optimum, bulky precipitates were obtained in the agglutination tubes, while at higher saturations anomalous precipitates occurred in the antigen-antiserum and antigen-normal serum tubes.

(iii) *Optimum Antigen Dilution.*—Clearest results were obtained at an antigen dilution of 1 : 4 with normal saline.

(iv) *Optimum Dialysis Time.*—Dialysis of antigen against tap water is considered to result in a loss of toxic constituents. When undialysed infective sap was used as antigen in the agglutination reaction, no precipitate could be observed, and, when dialysis was allowed to proceed for 2½, 3, or 3¼ hr, clearest results were obtained after 3¼ hr. No precipitate resulted after 2½ hr dialysis. It is evident that a substance which inhibits agglutination is present in undialysed sap, and such a substance may inhibit antibody production if undialysed sap is used as antigen for inoculation.

(v) *Antiserum Stability.*—Beemster and van der Want (1951) emphasize that antiserum to B.Y.M.V. is unstable and that serological activity is lost after 2 or 3 days storage at 1°C. This is suggestive of complement exerting a non-specific effect, but when antisera to both strains of P.M.V. were heated to 56°C for 30 min, following saturation, no loss in activity could be demonstrated.

(vi) *Determination of Titre.*—Antisera were diluted as shown in Table 1, and 1 : 4 dilution of antigen was used in each case. A positive reaction was characterized by the presence of a bulky precipitate in the bottom of each tube, and a clear solution above, in which a few white floccules could usually be observed (Fig. 1). This reaction was apparent 1–1½ hr after incubation.

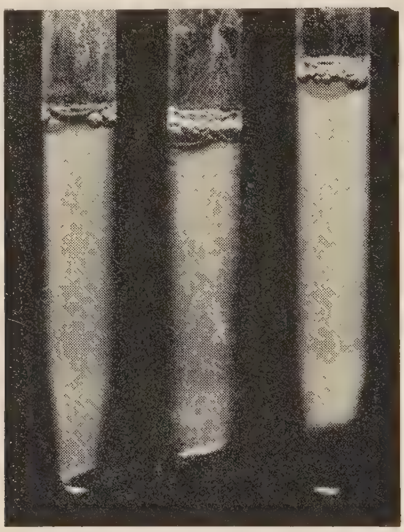

Fig. 1.—A positive agglutination test is indicated by the centre antigen-antiserum tube, which shows a clearing above the bulky precipitate and the presence of a few floccules, compared with the tube containing healthy sap–antiserum on the left, and with antigen–normal serum tube on the right. The healthy sap–normal serum tube is not shown but is similar in appearance to the tube on the left.

Thus the antiserum to B.Y.M.V. has an effective titre of 1 : 384, and effective titres of 1 : 576 and 1 : 704 to P.M.V. strain 1 and P.M.V. strain 2 respectively.

(*b*) *Serological Relationships*

(i) *Cross-agglutination Relations of the Viruses.*—Results of duplicate determinations are shown in Table 2. For each, a dilution series of saturated antisera from 1 : 1 to 1 : 16 (as shown in Table 1) was used.

Other field isolates whose host ranges corresponded to those of the antigens used for producing the antisera were also tested, viz. two of B.Y.M.V., one of P.M.V. strain 1 and two of P.M.V. strain 2. In each case the reactions were similar to those shown. Another strain of B.Y.M.V. (reported by Hutton and Peak 1954) also gave positive cross-agglutination reactions with all antisera.

(ii) *Cross-absorption Experiments.*—When B.Y.M.V. antiserum was saturated with P.M.V. strain 1 and P.M.V. strain 2 antigens, respectively, and the saturated antiserum then tested against B.Y.M.V. antigen, no positive agglutinations could be observed. Similarly, when P.M.V. strain 1 and P.M.V. strain 2 were saturated with their heterologous antigens, no agglutinations resulted when the homologous antigens were used to test serological activity.

(c) *Cross-protection Experiments*

Ideally, the challenging virus used in demonstrating mutual immunization should be one capable of producing local lesions in the host chosen (Bennett 1953). With P.M.V. and B.Y.M.V. this is not possible but the two may be separated by the inability of P.M.V. to infect beans (Goodchild 1956). Broad bean plants were inoculated with P.M.V. strains 1 and 2, the immunizing virus. When a basal shoot had developed two leaves with mottle symptoms, the main shoot was removed and these leaves inoculated with the challenging virus B.Y.M.V. Three weeks later the shoots were removed and the extracted sap from each used to inoculate six bean, var. Canadian Wonder, and six broad bean, var. Windsor Long Pod, plants. Of 24 plants with P.M.V. strain 1 and 36 plants with P.M.V. strain 2 treated in this way, in no case could the challenging virus be isolated, indicating an immunizing effect.

Table 2

SUMMARIZED RESULTS OF CROSS-AGGLUTINATION EXPERIMENTS

+ = Positive agglutination reaction

Antigen	Sera		
	Bean Yellow Mosaic Virus	Pea Mosaic Virus Strain 1	Pea Mosaic Virus Strain 2
Bean yellow mosaic virus	+	+	+
Pea mosaic virus strain 1	+	+	+
Pea mosaic virus strain 2	+	+	+

IV. Discussion

By the use of massive doses of P.M.V. antigen for inoculating the marginal ear vein of a rabbit and the appropriate conditions conducive to agglutination, it has been possible to demonstrate serological activity for this virus. The chief weakness in the technique lies in the preparation of antigen and the inability to provide sufficiently aseptic conditions, so that pathogenic organisms may enter, resulting in death of the animal. In practice, however, provided reasonable care is taken the chances of this occurring are relatively small. Anaphylaxis is also a danger, but is overcome to some extent by successive massive doses of antigen at relatively short time intervals, although in using crude plant sap there is a danger of the animal already having become sensitized through its feeding ration.

The methods used in demonstrating serological activity by agglutination have been described in as much detail as is possible, for conditions must be controlled to ensure the success of the technique. The results of the agglutination tests between B.Y.M.V. and P.M.V. strains 1 and 2 and their heterologous antisera indicate the two viruses to be serologically related. The degree of the relationship seems to be absolute, as no agglutinations could be effected after antisera had been saturated with heterologous antigens. However, as the virus appears to be present in low concentration in the host, the titre of such antisera, with respect to the homologous antigen after absorption by the heterologous antigen, may be so low that the relatively crude agglutination technique could not detect its presence.

The serological similarity of B.Y.M.V. and P.M.V. is supported by the results of cross-protection tests, which show a mutual immunizing effect between the two strains of P.M.V. and B.Y.M.V. strain 1.

Beemster and van der Want (1951) have shown bean mosaic and B.Y.M.V. to be serologically similar, and Grogan and Walker (1948) were able to show that the two viruses have a certain immunizing effect in french beans. The results reported here show a similar relationship between B.Y.M.V. and P.M.V. Van der Want (1954) has reported alfalfa mosaic, B.Y.M.V., and white clover mosaic as being serologically distinct and results of cross-protection tests show that these viruses do not exert an immunizing effect for each other.

Thus it is now possible to group the legume viruses with respect to their sero-serological properties, but the large group consisting of strains of bean mosaic virus, B.Y.M.V., and P.M.V. are shown to be serologically related. It is likely, however, that some strains of B.Y.M.V. and, in particular, the necrotic lesion-producing strain of Zaumeyer and Fisher (1953) may not be serologically related to the strain reported here and by Beemster and van der Want (1951) by virtue of their reactions on hosts outside the family Leguminosae and other divergent properties. To determine the presence of strains of viruses belonging to this broad group, then, it is still necessary to consider reactions on differential hosts (Goodchild 1956) as the main criteria for separation.

V. Acknowledgments

Grateful acknowledgment is made to Associate Professor N. H. White, for suggesting the project and for criticism and assistance throughout; to Associate Professor J. M. Vincent, for advice and for providing the facilities necessary to carry out the procedures involved; and to the Research Grants Committee of the University of Sydney for providing funds, in part, to carry out this project.

VI. References

Bawden, F. C. (1948).—Review of work on the serological reactions of plant viruses. Rep. Rothamst. Exp. Sta. pp. 120–7.

Beemster, A. B. R., and van der Want, J. P. H. (1951).—Serological investigations on the Phaseolus Viruses 1 and 2. *Leeuwenhoek Ned. Tijdschr.* **17**: 285–96.

Bennett, C. W. (1953).—Interactions between viruses and virus strains. In "Advances in Virus Research". Vol. 1. (Academic Press Inc.: New York.)

Chester, K. S. (1937).—A simple and rapid method for identifying plant viruses in the field. *Phytopathology* **27**: 722–7.

GOODCHILD, D. J. (1956).—Relationships of legume viruses in Australia. I. Strains of bean yellow mosaic virus and pea mosaic virus. *Aust. J. Biol. Sci.* **9**: 213–30.

GROGAN, R. G., and WALKER, J. C. (1948).—Interrelation of bean virus 1 and 2 as shown by cross-protection tests. *Phytopathology* **38**: 489–93.

HUTTON, E. M., and PEAK, J. W. (1954).—Varietal reactions of *Trifolium subterraneum L.* to Phaseolus virus 2 Pierce. *Aust. J. Agric. Res.* **5**: 596–607.

MURPHY, D. W., and PIERCE, W. H. (1937).—Common mosaic of the garden pea *Pisum sativum*. *Phytopathology* **27**: 711–21.

STUBBS, M. W. (1937).—Certain viruses of the garden pea. *Phytopathology* **27**: 242–66.

VAN SLOGTEREN, E. (1955).—Serological diagnosis of plant virus diseases. *Ann. Appl. Biol.* **42**: 122–8.

VAN DER WANT, J. P. H. (1954).—Onderzoekingen over Virusziekten van de Boon (*Phaseolus vulgaris* L.). D.Sc. Thesis, Wageningen.

ZAUMEYER, W. J., and FISHER, H. H. (1953).—A new necrotic lesion-producing strain of yellow bean mosaic. *Phytopathology* **43**: 45–9.

OXIDATION OF MANGANESE BY SOIL MICROORGANISMS

By S. M. Bromfield*

[*Manuscript received September* 20, 1955]

Summary

Oxidation of manganous sulphate by an association of two bacteria and by a newly-isolated strain of a *Corynebacterium* sp. was studied in an artificial medium containing 0·005 per cent. yeast extract, 0·005 per cent. manganous sulphate, and various other inorganic salts.

The organisms produced an insoluble manganese oxide which was intimately mixed with bacterial cells. Oxidation of manganese occurred in growing cultures of the new isolate just after the organism reached the stationary phase in its growth-cycle and it appeared that the organism did not grow autotrophically during the period of oxidation. This period coincided with the maximum oxidizing activity of cells grown in manganese-deficient medium. Cells harvested from this medium were readily able to oxidize a 0·005 per cent. manganous sulphate solution; this reaction was inhibited by boiling and by various poisons, especially catalase inhibitors and hydrogen peroxide, and was not accelerated by any of the substrates tested. The mechanism of oxidation is discussed.

I. Introduction

Manganous sulphate is readily oxidized when it is added to neutral soils (Gerretsen 1937; Leeper and Swaby 1940; Mann and Quastel 1946). This oxidation is the result of the activity of soil microorganisms (Gerretsen 1937; Bromfield and Skerman 1950; Timonin 1950*b*).

Manganese-oxidizing organisms have been isolated from soil using various media. Söhngen (1914) used a medium containing calcium malate, and later, Gerretsen (1937), McLachlan (1941), and Timonin (1946) used a medium containing calcium citrate. Söhngen concluded that malate and other hydroxyacids, including citrate, catalyse the oxidation of manganese and that the reaction is favoured by an increase in alkalinity. He suggested that the microbial oxidation of manganese in the presence of citrate is due to the production of an alkaline pH, which accelerates this catalytic oxidation.

Bromfield and Skerman (1950) showed that many organisms which oxidize manganese in the presence of citrate cannot oxidize manganese in soil, and they concluded that a medium containing citrate was undesirable for isolating the manganese-oxidizing organisms active in soil. They used a soil-water-extract agar in preference to the citrate medium and isolated a pair of bacteria which could oxidize manganese added to soil. Others have also observed oxidation in media which do not contain citrate. Beijerinck (1914) used a manganous carbonate agar, Thiele (1925) employed peat extract and low concentrations of peptone, and Timonin (1950*a*, 1950*b*) observed oxidation in the presence of agar alone and with various nitrogen sources. Numerous fungi and some bacteria have been isolated on these non-citrate media, but these isolates have not been studied in detail, and the mechanism of oxidation is still obscure. Beijerinck (1914) suggested that the oxidation resulted from autotrophic

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growth. Waksman (1940) and Starkey (1955), however, concluded that this question was still unanswered. Beijerinck (1914) and Thiele (1925) observed that the oxide was deposited outside the mycelia of oxidizing fungi, and postulated that an extracellular enzyme was involved. Beijerinck (1914) claimed that the oxidation product was manganese dioxide.

This paper describes the preparation of a medium suitable for studying manganese oxidation, factors affecting oxidation by pure cultures of bacteria, and a study of the mechanism of oxidation.

II. Media and Methods

Mixed cultures of species of *Corynebacterium* and *Chromobacterium*, previously studied by Bromfield and Skerman (1950), were grown in a manganese medium of the following composition:

Distilled water	100 ml	$Ca_3(PO_4)_2$	0·010 g
KH_2PO_4	0·005 g	"Difco" yeast extract	0·005 g
$MgSO_4.7H_2O$	0·002 g	$MnSO_4.4H_2O$	0·005 g
$(NH_4)_2\,SO_4$	0·010 g		

The pH of the medium was adjusted to 6·0 and was autoclaved at 10 lb/sq. in. for 15 min. The medium was also used in a solid form after the inclusion of 2 per cent. "Difco" agar.

This manganese medium was preferred to the soil-extract manganese medium of Bromfield and Skerman (1950) because it was more defined and easier to prepare. The manganese remained unoxidized in the sterile medium and in this regard it is to be preferred to a citrate medium since oxidized manganese can be detected in the latter.

The liquid medium was dispensed in either $6\times\frac{3}{4}$ in. test tubes or 1-l. beakers and inoculated with one loopful or 1 ml, respectively, of a suspension of a 24-hr culture grown on nutrient agar (0·5 per cent. sucrose, 0·3 per cent. "Difco" yeast extract, and inorganic salts as above). The inoculated medium was incubated at 27°C for periods up to 21 days during which time a dark brown deposit was formed which gave a deep blue colour with 1 per cent. (w/v) benzidine hydrochloride solution in 2N acetic acid, indicating that the manganese had been oxidized.

Determinations of pH were made with a glass electrode. Manganese determinations were made colorimetrically by oxidizing manganous ion to permanganate with periodate. Manganese in the deposit was reduced to manganous ion with sodium sulphite and sulphuric acid before the addition of periodate. The permanganate colour was measured in a "Unicam" spectrophotometer at 530 $m\mu$.

Viable cell counts were determined by mixing serial 10-fold dilutions of the culture with nutrient agar and counting the number of colonies which developed after 48 hr.

Total cell counts were made on samples of 5 ml of culture stained with 1 drop of 1 per cent. aq. Victoria blue 4R. In oxidizing cultures the oxide cementing material was dissolved with acidified sodium sulphite solution before staining. The cells were then counted on a haemocytometer slide.

Cell suspensions which oxidize manganese solutions were prepared using the cells harvested by centrifugation from cultures grown in a manganese free medium. The cells were suspended in distilled water to give a suspension 50-fold more concentrated than the original culture. A cell suspension prepared in this way oxidized manganese within 5 min at 35°C. Attempts to obtain large amounts of active cell material from the growth which occurred on ordinary nutrient media failed. Activity of cell suspensions was determined by mixing 1 ml of the suspension with 1 ml of 0·01 per cent. (w/v) manganous sulphate solution in a $6 \times \frac{3}{4}$ in. test tube, incubating in a water-bath at 40°C, and noting the time for the appearance of manganese oxide, or alternatively, noting the relative amount of oxidation after a given time.

III. Experimental Results

(a) *Experiments with Manganese Medium*

The results of the experiments about to be described formed the basis for the composition of the manganese medium used in this investigation.

Table 1

EFFECT OF VARIOUS CONCENTRATIONS OF MANGANESE AND YEAST EXTRACT ON THE TIME FOR THE APPEARANCE OF, AND DEGREE OF, MANGANESE OXIDATION

N = No oxidation after 21 days; P = poor oxidation; G = good oxidation

Concentration of Yeast Extract (g %)	Time in Days for, and Degree of, Oxidation							
	Concentration of $MnSO_4.4H_2O$ (g %)							
	0·00	0·001	0·005	0·01	0·02	0·03	0·05	0·1
0·00	N	N	N	N	N	N	N	N
0·001	N	6, P	6, P	6, P	6, P	6, P	N	N
0·005	N	1, P	1, G	1, G	2, G	6, G	16, P	N
0·01	N	1, P	1, G	1, G	2, G	6, G	16, P	N
0·5	N	N	N	N	N	N	N	N

(i) *Effect of Varying the Composition of the Manganese Medium on the Oxidation of Manganese.*—The concentrations of manganous sulphate and "Difco" yeast extract were varied over a range of 0–0·1 per cent. (w/v) and 0–0·5 per cent. (w/v) respectively. The effect this variation had on oxidation is given in Table 1.

Most rapid and complete oxidation of manganese occurred in the media which contained 0·005–0·01 per cent. manganous sulphate and 0·005–0·01 per cent. yeast extract. With higher or lower concentrations of either of these substances oxidation was retarded or did not occur at all. Oxidation was absent in media without yeast extract and in media containing 0·5 per cent. yeast extract or 0·1 per cent. manganous sulphate. The inhibition of oxidation in media containing 0·1 per cent. manganous sulphate was due to inhibition of growth. In the media containing noninhibitory amounts of manganous sulphate and 0·5 per cent. yeast extract, growth was very good but oxidation of manganese could not be detected. A concentration of 0·005 per cent. (w/v) for both manganous sulphate and "Difco" yeast extract was used in the manganese medium in further experiments.

Calcium phosphate was replaced by other sources of calcium in equivalent amounts and it was found that oxidation occurred equally well with calcium carbonate, decreased with calcium chloride (pH of medium adjusted to 6·0 with sodium hydroxide), and did not occur at all with calcium chloride (pH 5·3). When ammonium sulphate was replaced by ammonium nitrate or sodium nitrate the oxidation of manganese decreased. The omission of one or other of the inorganic salts from manganese medium either prevented oxidation or delayed its onset 7–8 days With the complete inorganic salt complement oxidation commenced within 24 hr.

To determine at what pH values oxidation occurred in this medium, the pH was adjusted with 0·1N hydrochloric acid, and 0·1N sodium hydroxide to give a pH range of 3·7–8·4. The media were inoculated and incubated for 1 week, after which the cultures were examined for oxidation and growth and final pH values were determined. Oxidation occurred in media with an initial pH value of between 4·2 and 7·0, but not at 3·7 or above 7·6. During growth the pH increased by 0·6 of a unit in the more acid media, and decreased by 0·7 of a unit in the alkaline media. In media with an initial pH of 5·4 there was no change in pH during oxidation. Growth was comparable at all pH values except below 4 and above 8, in which cases it was poorer. It appears that a pH value between 5 and 6 is most suitable for oxidation in this medium.

"Difco" yeast extract was replaced in liquid manganese medium by the organic substances listed in Table 2. The final concentration of organic substance was 0·005 per cent. and the pH of the medium adjusted to 6·0. The relative amount of oxidation after 1, 3, and 21 days is also given in the table.

Oxidation was greatest in the "Difco" yeast extract, glucose, and mixed amino acid media but oxidation occurred more rapidly in the first. Slight to moderate oxidation occurred when various amino acids, α-ketoglutaric acid, and pyruvic acid were present, but other amino acids and organic acids were ineffective. The mineral-salts control also gave no oxidation. It was found subsequently that the addition of various growth factors and vitamins with the above substrates had no stimulating effect on the oxidation of manganese by the bacteria. It is of interest to note at this stage that oxidation occurred after 14 days on a mineral-salts control medium solidified with agar. This means that agar itself provided sufficient substrate to enable the organisms to oxidize manganese.

(ii) *Behaviour of Manganese-oxidizing Bacteria Isolated on Gerretsen's Medium.*—To determine whether these bacteria could also oxidize manganese in the "new" manganese medium, previously-isolated manganese-oxidizing bacteria (Bromfield and Skerman 1950) were plated on it. Of the six bacteria tested only one was able to oxidize on the new medium. This indicates that it is more selective than Gerretsen's and that oxidation on it may be a specific property of the bacterium and not due merely to the production of an alkaline pH.

(*b*) *Studies on the Relationship between* Corynebacterium *sp. and* Chromobacterium *sp. in the Oxidation of Manganese*

It has been shown (Bromfield and Skerman 1950) that oxidation of manganous sulphate occurs in a mixed but not in a single pure culture of the above two organisms. Experiments were made to find whether this was due to the production of a readily diffusible substance by one organism, which enabled the other to oxidize manganese.

TABLE 2

RELATIVE AMOUNTS OF MANGANESE OXIDE PRODUCED IN LIQUID MANGANESE MEDIUM CONTAINING VARIOUS SUBSTRATES

– = No oxidation; + = faint deposit, light brown; ++ = moderate deposit, brown; +++ = heavy deposit, dark brown

Organic Substance (0·005%)	Relative Amounts of Oxide after:		
	1 Day	3 Days	21 Days
"Difco" yeast extract	++	+++	+++
Davis gelatin	+	+	++
Glucose	–	++	+++
DL-Alanine	–	+	+
DL-Serine	–	–	∓
DL-Aminobutyric acid	–	–	–
DL-Threonine	–	–	–
DL-Valine	–	–	–
L-Leucine	+	+	±
L-Asparagine	+	+	+
L-Glutamic acid	+	+	++
L-Lysine HCl	–	+	+
L-Histidine HCl	–	+	+
L-Cystine	–	–	–
DL-Methionine	–	–	–
DL-β-Phenylalanine	–	+	++
L-Tyrosine	+	+	++
L-Tryptophan	–	–	–
Mixed amino acids	++	++	+++
α-Ketoglutaric acid	–	+	++
Pyruvic acid	–	–	+
Citric acid	–	–	–
Succinic acid	–	–	–
Fumaric acid	–	–	–
Malic acid	–	–	–
No substrate	–	–	–

Cultures of the two organisms were grown separately in 10 ml of manganese medium for 3 days at 27°C; they were then either heated at 70°C for 3 min, filtered through a Seitz sterilizing filter, and treated with 1 ml ether, or left untreated and then re-inoculated with the other bacterium. After 10 days at 27°C only the untreated, doubly-inoculated cultures gave oxidation. Sterility checks showed that in all the treated tubes only the organism used for the second inoculation was present.

Following this experiment, the two bacteria were grown in the same medium but in such a way that pure cultures were separated from one another by a collodion membrane. In this case oxidation did not occur unless both organisms were present together on one side of the membrane. It was shown that the constituents of the medium could diffuse through the membrane. This was done by separating the sterile medium from doubly-inoculated distilled water. In this case oxidation occurred in the side containing the distilled water.

In order to determine whether active growth by both organisms was necessary for oxidation, the two species were grown successively on agar plates. The first organism was allowed to grow for 3 days and then treated with 1 ml ether. After the ether had evaporated the plates were re-inoculated with the second organism and incubated for a further 3 days. The ether treatment did not kill all the organisms and, as a result, oxidation occurred on the doubly-inoculated plates but only where the surviving organisms developed. A microscopic examination of these sites of oxidation revealed that the oxide was dense and confined within the surviving *Corynebacterium* colonies, but was only scattered thinly above the *Chromobacterium* colonies. The deposition of oxide in *Corynebacterium* colonies could be demonstrated in another way by streaking the two organisms in parallel lines on the same medium. The *Chromobacterium* sp. due to its spreading habit of growth on manganese medium, eventually overgrew the *Corynebacterium* colonies and where this happened oxidation occurred (Plate 1, Fig. 1). This figure also illustrates how intimate is the association of the two organisms leading to oxidation, a colony of *Corynebacterium* sp. which is three-quarters covered by the spreading *Chromobacterium* sp. shows oxidation only in that portion.

To determine whether mixtures of cell suspensions of *Corynebacterium* sp. and *Chromobacterium* sp. could oxidize a manganous sulphate solution, these organisms were grown in pure and mixed culture in manganese-deficient medium. The cells were harvested by centrifugation when a manganese medium control started to show oxidation. Four cell suspensions were prepared from the harvested cell material: (1) a suspension of *Corynebacterium*, (2) a suspension of *Chromobacterium*, (3) a mixed suspension prepared from the above two, and (4) a suspension of the growth from the mixed culture. These suspensions were corrected for dilution and incubated at 35°C with 0·005 per cent. (w/v) manganous sulphate. After 1 hr incubation, only suspension (4) produced oxidation. This result also indicates that the two organisms have to be growing together to produce the oxidizing system.

(*c*) *Isolation of a Single Pure Culture of a Bacterium Capable of Oxidizing Manganese in Manganese Medium*

It was observed on plates of manganese medium inoculated from single colonies of the *Corynebacterium* sp. (subsequently referred to as strain A) that a few single colonies showed segments which contained manganese oxide (Plate 1, Fig. 2). On plating these segments, completely brown and completely white colonies developed (Plate 1, Fig. 2). The brown colonies, which will be called strain B, usually gave colonies on re-plating which were completely brown, although sometimes white segments or wholly white colonies developed, especially in old cultures (14 days) (Plate 1, Fig. 4). These white colonies were shown to be similar to strain A. Strain A and strain B were subjected to the standard biochemical tests used for identification and were found to differ in these tests only in their ability to oxidize manganese. Strain B when inoculated into sterile soil alone and treated according to Gerretsen's technique (Gerretsen 1937) produced a brown-ring deposit of manganese oxide.

(*d*) *Oxidation of Manganous Sulphate by Growing Cultures of* Corynebacterium *Strain B*

Experiments were next undertaken to determine the extent of oxidation which occurred in growing cultures of strain B, and to find at what stage during its growth oxidation took place.

(i) *Extent of Oxidation*.—To determine the amount of oxide which had formed at various times during the growth of strain B, the amounts of manganese in solution and in the deposit were measured at 2-hourly intervals over a period of 48 hr. The inoculum used was 1 ml of suspension per 500 ml of medium. The results of these determinations are presented in Figure 1.

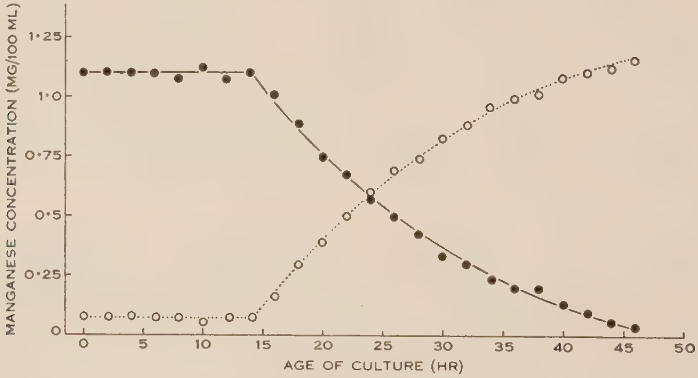


Fig. 1.—Rate of oxidation of manganous sulphate in manganese medium by *Corynebacterium* strain B. ● Mn^{++} in solution. ○ Mn^{++} in deposit after reduction. Mn determined as permangate.

Oxide commenced to form after an initial incubation period of 15 hr. The rate at which it formed declined progressively during the course of the experiment and after about 48 hr oxidation was virtually complete. Qualitative tests for higher valency manganese were made on the supernatant of centrifuged samples during the experiment, to see if oxidized manganese was in solution. However, this could not be demonstrated with benzidine hydrochloride.

(ii) *Nature of Growth Curve during Oxidation*.—To ascertain at what stage during the growth of the culture oxidation commenced, viable cell counts were made on the supernatant of cultures similar to the above. The results of these counts are presented in Figure 2 together with a brief description of the appearance of the culture at arbitrary stages indicated in the figure by the letters A to H.

The pertinent observation is that the appearance of oxide in the culture occurred just after the onset of the stationary phase in the growth curve, i.e. after 18- to 20-hr incubation. This apparently coincides with the onset of the active oxidative period determined in the previous experiment.

(iii) *Examination of the Apparent Fall and Rise in the Number of Viable Cells during Oxidation.*—The dip during the interval D to F (Fig. 2) was confirmed in other experiments although its magnitude was not constant. It was of special interest since the rise might have been due to autotrophic growth. If cell multiplication had taken place during this period there should be approximately twice as many cells in the culture at F as at D. Total cell counts at C and at G were next undertaken to determine whether multiplication had, in fact, taken place. The experimental procedure was modified to overcome the interference of calcium phosphate particles in the direct counting. The cultures were grown for 17 hr in the complete medium at which time the supernatant was transferred to sterile 1-l. beakers and given further incubation. Direct counts were made of the number of oxide clumps and free cells in the suspended culture and also of the total number of cells after removal of oxide. Counts of viable cells were also made at C and G. For comparison, manganese-deficient medium was also examined in the same way. The various determinations are presented in Table 3.

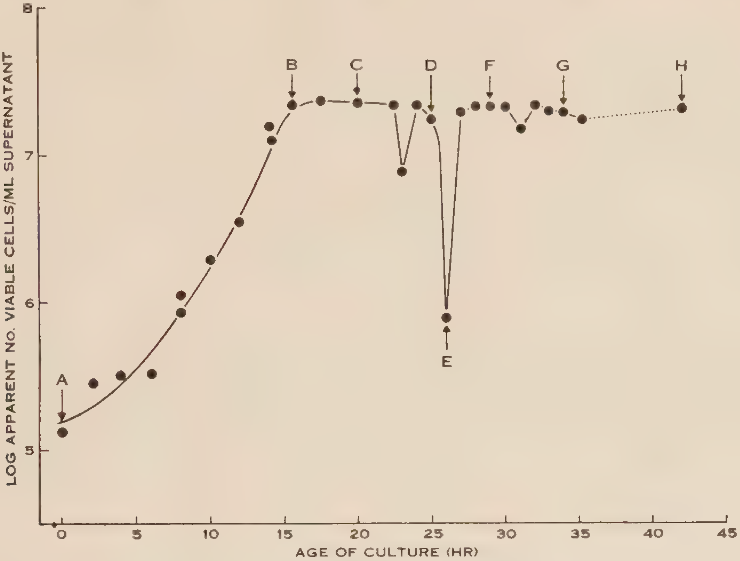


Fig. 2.—Number of viable cells in supernatant of a culture of *Corynebacterium* strain B growing in manganese medium. Appearance of the culture: *A-B*, increasing milky turbidity; *B-C*, culture becomes very pale yellow; *C-D*, culture light brown with small brown particles in suspension, slight deposit; at *E*, light brown, fairly large brown particles in suspension which are visibly settling out, moderate deposit; *E-F*, increasing deposit, supernatant lighter in colour but still containing suspended brown particles; *F-G*, supernatant turbid, milky with very few brown particles, heavy dark brown deposit; at *H*, supernatant turbid, milky, no obvious brown particles in suspension, heavy dark brown deposit.

The counts show that the total numbers of cells in the manganese medium did not increase greatly during the course of the oxidation and were virtually the same as the total number of cells in the manganese-free medium. The organism, therefore, apparently does not derive energy for growth during the oxidation of manganese: this energy must come wholly from the yeast extract. The fact that there was no gross change in the total number of cells indicates that the dip in viable cells during the interval D to F was an apparent one, due possibly to the adsorption and subsequent release of large numbers of viable cells by the surface of the oxide clumps. The observed fall in the number of viable cells (Table 3) is attributed to the increased aeration the cultures received during transferring and suspending procedures.

Table 3

NUMBERS OF OXIDE CLUMPS, CELLS IN SUSPENSION, TOTAL CELLS, AND VIABLE CELLS OF CORYNEBACTERIUM STRAIN B GROWN IN MANGANESE-DEFICIENT AND MANGANESE MEDIA

Age of Culture (hr)	Stage (see Fig. 2)	Numbers/Ml ($\times 10^{-5}$)					
		Manganese Medium				Manganese-deficient Medium	
		Oxide Clumps	Cells in Suspension	Total Cells	Viable Cells	Total Cells	Viable Cells
17	*C*	4	287	323	201	334	195
41	*G-H*	8	228	375	64	370	104

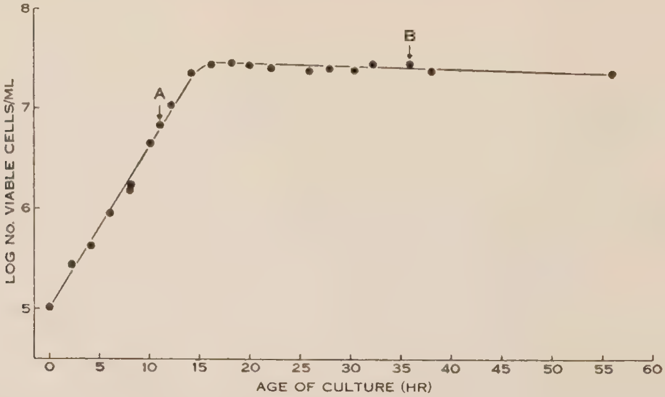


Fig. 3.—Number of viable cells in supernatant of a culture of *Corynebacterium* strain B grown in manganese-deficient medium. See also Table 4 for description of oxidizing activity of cells harvested during period *A-B*.

(iv) *Nature of the Oxide Deposit.*—A microscopic examination of the brown material which formed in the above cultures revealed that individual cells ($1 \times 2\ \mu$) first became coated with a thin layer of brown material, these cells then formed aggregates (about 10 μ in diameter) which in their turn came together and formed a loose sponge-like deposit (Plate 2, Figs. 1, 2, and 3). The precipitate is thus an intimate mixture of bacterial cells and manganese oxide.

(e) *Manganese Oxidation with Cell Suspensions*

In an attempt to elucidate the nature of the oxidation, experiments were undertaken with cell suspensions.

(i) *Age of Culture.*—To determine at what stage of growth strain B exhibited maximum oxidizing activity, its cells were harvested and tested at various stages during its growth. Activity was measured by noting whether oxidation had taken place after 5, 15, and 30 min. Viable cell counts were made at the time of sampling and the cells from the centrifuged sample were tested both as fresh cells and after allowing them to stand for 30 min. The results of this experiment are illustrated in Table 4 for the period of growth between A and B in Figure 3. The manganese-oxidizing activity of the cells coincided with the onset of the stationary phase and first appeared 18 hr after inoculation with a viable cell count of $2{\cdot}8 \times 10^7$ cells per ml. The activity of the harvested cells increased to a peak during the next 8 hr and then lost activity until, at 36 hr, activity was almost nil. The activity of the cells at 18, 20, 22, and 24 hr was increased by allowing them to stand for 30 min at room temperature before testing. After 24 hr the activity did not increase on standing. These results indicate that to obtain cell suspensions with good oxidizing activity the cells should be harvested 2–8 hr after the onset of the stationary phase and allowed to stand for 30 min.

TABLE 4

OXIDIZING ACTIVITY OF CELLS HARVESTED FROM MANGANESE-DEFICIENT MEDIUM AT VARIOUS STAGES OF GROWTH OF CORYNEBACTERIUM STRAIN B

— = No oxidation; ± = query oxidation; + = oxidation

Stage (see Fig. 3)	Age of Culture (hr)	Fresh Cells			Cells Rested 30 Min		
		Oxidation after Incubation with Manganese for:			Oxidation after Incubation with Manganese for:		
		5 Min	15 Min	30 Min	5 Min	15 Min	30 Min
A	11	—	—	—	—	—	—
	14	—	—	—	—	—	—
	16	—	—	—	—	—	—
	18	—	—	+	—	+	+
	20	—	—	+	+	+	+
	22	—	+	+	+	+	+
	24	—	+	+	+	+	+
	26	+	+	+	+	+	+
	28	+	+	+	+	+	+
	30	—	+	+	—	±	+
	32	—	+	+	—	+	+
	34	—	—	+	—	—	+
B	36	—	—	—	—	—	—

(ii) *Effect of Temperature.*—To determine whether cell suspensions of cultures grown at 27°C could oxidize manganese at temperatures which inhibited oxidation by growing cultures, cell suspensions and inoculated medium were incubated at 27, 35, 45, and 100°C. Oxidation occurred in cell suspensions at all temperatures except 100°C, whereas the inoculated medium only gave oxidation at 27°C; at 35°C the cultures grew sparsely, and at 45 and 100°C the inoculum was killed. The fact that cells from cultures grown at 27°C can oxidize manganese at 45°C but not at 100°C suggests that a heat labile enzyme system is involved.

(iii) *Addition of Organic Substances.*—It was thought that manganese oxidation might occur concurrently during the oxidation of an organic substance, or that the manganous ion by itself, could act as an electron donor. To invesitgate these possibilities, oxidation by cell suspension was studied in the presence of the following oxidizable organic substances:

L-Lactate
Citrate
Succinate
Malate
Pyruvate
Fumarate
DL-Histidine
L-Histidine
L-Phenylalanine
L-Arginine
L-Leucine
L-Methionine
DL-Phenylalanine
L-Tyrosine
L-Glutamate
Yeast extract
Glucose
p-Cresol

All the substances were used as neutral 0·005 per cent. (w/v) solutions; glutamate and yeast extract were also used at a concentration of 0·05 per cent. The test system contained 1 ml of substrate solution, 1 ml cell suspension, and 1 ml 0·01 per cent. manganous sulphate. Oxidation occurred in 10 min with all substances except 0·05 per cent. yeast extract, L-arginine, and L-histidine. The water control gave as good, or better, oxidation than did any of the substances tested. It was thought that the inhibition of oxidation by L-arginine and L-histidine might have been due to their preventing, by competition, electron transfer from manganous ion to an electron acceptor, i.e. the amino acids themselves might have given up an electron more readily than the manganous ion. To test this, the amino acid oxidase activity of the cell suspension in the presence of 0·005 per cent. L-arginine, L-histidine, L-methionine, L-phenylalanine, and L-leucine was determined by Thunberg's methylene blue reduction technique. The amino acid oxidase activity of the cell suspension was found to be slight; the times for complete reduction of 1/6000 methylene blue at 37°C being 2 hr or more in all cases: L-methionine, 120 min; L-leucine, 116 min; L-phenylalanine, partial reduction after 120 min; L-histidine, partial reduction after 120 min; L-arginine and water control, no reduction after 120 min. That somewhat greater amino acid oxidase activity occurred in the presence of L-methionine and L-leucine than with L-arginine and L-histidine suggests that the observed inhibition of manganese oxidation is due to some cause other than electron competition. It is concluded from these results that the complete oxidizing system is present in the cell suspension, and that it is unlikely that L-amino oxidase or the intermediate acid dehydrogenases tested are involved in the electron transfer when manganous ion is oxidized.

(iv) *Effect of Enzyme Poisons.*—In an attempt to elucidate the nature of the system responsible for the oxidation of manganese the effects of various poisons on the oxidizing activity of cell suspensions were studied. The poisons used and their effect on oxidation are listed in Table 5. They were added directly to a mixture of 1 ml of cell suspension and 1 ml of 0·01 per cent. manganous sulphate and incubated for various intervals. Mercuric chloride, copper chloride, potassium cyanide, and sodium azide completely inhibited oxidation at certain concentrations as also did benzene treatment and boiling. The other substances, with the exception of *n*-octyl alcohol and carbon monoxide, partially inhibited oxidation.

TABLE 5

EFFECT OF VARIOUS POISONS AND TREATMENTS ON THE OXIDATION OF MANGANESE BY CELL SUSPENSIONS OF CORYNEBACTERIUM STRAIN B

Treatment	Final Concentrations	Incubation Time (min)	Oxidation*
Control	—	10	++
Boiled cells	—	24 Hr	−
$HgCl_2$	M/2700	24 Hr	−
$CuCl_2$	M/300	24 Hr	−
$CuCl_2$	M/2000	1 Hr	+
$MnSO_4$	M/4500	10	++
$MnSO_4$	M/450	10	++
$MnSO_4$	M/225	10	+
$MnSO_4$	M/45	10	±
CO in dark	—	10	++
KCN	M/13000	10	+
KCN	M/1300	10	+
KCN	M/650	10	±
KCN	M/65	10	−
NaN_3	M/13000	10	+
NaN_3	M/1300	10	−
H_2O_2	M/400	10	++
$KCN+H_2O_2$	M/13000+M/400	10	−
$NaN_3+H_2O_2$	M/13000+M/400	10	−
Ethanol	33% v/v	20	+
Methanol	33% v/v	20	±
Benzene	Sat. soln.	20	−
Toluene	Sat. soln.	20	+
CCl_4	Sat. soln.	20	++
Chloroform	Sat. soln.	20	++
n-Octyl alcohol	Sat. soln.	20	++

* Colour relative to control produced on addition of 1 drop benzidine reagent to cell system.

These results again indicate that dehydrogenases and L-amino oxidase are not involved in the oxidation since it occurred in the presence of chloroform, toluene, and *n*-octyl alcohol. The inhibition by copper and mercury salts confirms the enzymatic nature of the oxidation. The inhibition by azide and cyanide suggest that a heavy metal-containing enzyme is involved. The absence of inhibition by carbon monoxide, in the dark, indicates that cytochrome oxidase is not part of the oxidation system.

It is of interest to note also that low concentrations of cyanide and of azide, which by themselves result in slight inhibition, can, in the presence of 0·0025M hydrogen peroxide, itself a slight inhibitor, completely inhibit oxidation. It was possible that this inhibition was due to catalase inactivation and that the hydrogen peroxide persisted and adversely affected the oxidizing system.

(v) *Effect of* 2, 4-*Dichlorophenol*.—To see whether catalase was involved in the oxidizing system, 2, 4-dichlorophenol, a specific inhibitor for catalase (Goldacre and Galston 1953), was added to cell suspensions to give final concentrations of 1×10^{-3}, 1×10^{-4}, and 1×10^{-5}M. Oxidation was adversely affected at all concentrations and was completely inhibited at the highest. This result suggested that catalase was involved in the oxidizing system.

(vi) *Tests for Catalase and Peroxidase*.—Since oxidation was affected by poisons which inhibit catalase and possibly other heavy metal-containing enzymes, tests were made for these two enzymes. Catalase was readily demonstrated in suspensions of cells. Peroxidase however could not be detected in either whole cells, ground cells, or cells incubated with toluene. Saturated aqueous guaiacol solution was used as the peroxidase substrate and the final concentration of peroxide was 0·025M.

(vii) *Effect of Hydrogen Peroxide on the Oxidation Product*.—It is known that hydrogen peroxide can reduce manganese oxides. To see if the inhibitory effect of hydrogen peroxide could be due to it reducing the oxidation product, 4-ml samples of a light suspension of oxide, collected from oxidizing cultures of strain B, were mixed with 1 ml of 0·05, 0·005, or 0·0005M hydrogen peroxide solution and allowed to stand for 20 min at 30°C. It was found that hydrogen peroxide at final concentrations of 0·01 and 0·001M completely reduced and dissolved the oxide suspension and at 0·0001M caused partial reduction. It was found also that this reduction could be prevented, or decreased, by adding a cell suspension of strain B showing good catalase activity, or by adding a purified catalase preparation to the oxide suspension.

It appears that the inhibition of oxidation by hydrogen peroxide could be due to peroxide preventing the accumulation of oxidized manganese, by reducing it as fast as it is formed.

IV. DISCUSSION

The inhibition of manganese oxidation by moderately high concentrations of yeast extract (Table 1) confirms a similar observation by Beijerinck (1914). The reason for this inhibition is not known but it appears that oxidation only occurs when the organism is grown under starvation conditions. It is possible that oxidation of manganese in soil could be inhibited by the addition of organic materials which enhance the growth of the organisms.

Mann and Quastel (1946) suggested that the oxidation of manganese in unsterile soil was due to actively proliferating microorganisms since the oxidation curve was similar to a growth curve. The results of the present work indicate, however, that oxidation only occurs when the organism is in the stationary phase (Figs. 1, 2, and 3; Table 4).

Beijerinck (1914) proposed that the oxidation of manganese provided energy for the autotrophic growth of the oxidizing organism. The bacterium studied in the present investigation does not appear to grow autotrophically since it does not grow in the absence of an organic substrate (Table 2), and since the total number of cells which grow in the manganese medium and in the manganese-deficient medium are virtually the same and do not increase significantly during oxidation (Table 3).

The mechanism of oxidation by mixtures of *Corynebacterium* strain A and *Chromobacterium* sp. was not unravelled, but it was shown that they have to be growing together to cause oxidation, and that strain A was the main member of the pair since it gave rise to a strain which could oxidize manganese in pure culture (Plate 1, Figs. 2–4). It may be that the *Chromobacterium* sp. induces the formation of, or supplements, an enzyme system in strain A.

The studies on the mechanism of manganese oxidation by *Corynebacterium* strain B revealed that an intracellular enzyme system was involved. This system is different from the manganese-oxidizing peroxidase system described by Kenten and Mann (1950) since peroxidase activity could not be detected in cell suspensions and since hydrogen peroxide, in the absence of catalase, inhibited oxidation. The observed inhibition by peroxide and the need for catalase in the oxidizing system coincides with a similar observation by Andreae (1955). He studied the photo-induced oxidation of manganese in a system containing manganese, pyrophosphate, riboflavin, a hydrogen donor such as *p*-cresol, catalase, and air, and found that the catalase was necessary to decompose the hydrogen peroxide which formed when reduced riboflavin was reoxidized. He postulated that the manganese was oxidized by the oxidation product of the hydrogen donor. It is tempting to suggest a system similar to that of Andreae to account for the need for catalase in *Corynebacterium* strain B. However, the failure of various organic hydrogen donors, including *p*-cresol, to enhance oxidation by cell suspensions, together with the observation that the activity of cells often improved on standing, suggests that oxidation proceeds without an organic hydrogen donor. If this is so then it is likely that manganese itself is the specific electron donor in the oxidizing system. To decide this issue it will be necessary to use cell free preparations.

Since oxidized manganese was not detected in the supernatant of centrifuged oxidizing cultures it is probable that the manganese is oxidized inside the cell and that it deposits as the oxide on the surface of the cells as it diffuses out. The manganous ion may first form an organic complex, perhaps with the enzyme itself, which is more easily oxidized than the free manganous ion (Martell and Calvin 1952, p. 58). It is proposed to investigate the oxidation product and determine its availability to plants.

V. ACKNOWLEDGMENTS

The author wishes to thank the following members of the Division of Plant Industry, C.S.I.R.O.: Mr. J. Pomeroy for the photomicrographs, Mr. W. Goodwin for the preparation of the figures, and Mrs. H. Mykytowycz for her technical assistance.

VI. REFERENCES

ANDREAE, W. A. (1955).—The photo-induced oxidation of manganous ions. *Arch. Biochem. Biophys.* **55**: 584–6.

Beijerinck, M. W. (1914).—Oxidation of manganous carbonate by microorganisms. *Versl. Akad. Wetensch.* **22**: 415–20.

Bromfield, S. M., and Skerman, V. B. D. (1950).—Biological oxidation of manganese in soils. *Soil Sci.* **69**: 337–47.

Gerretsen, F. C. (1937).—Manganese deficiency of oats and its relation to soil bacteria. *Ann. Bot.* (N.S.) **1**: 207–30.

Goldacre, P. L., and Galston, A. W. (1953).—The specific inhibition of catalase by substituted phenols. *Arch. Biochem. Biophys.* **43**: 169–75.

Kenten, R. H., and Mann, P. J. G. (1950).—Oxidation of manganese by peroxidase systems. *Biochem. J.* **46**: 67–73.

Leeper, G. W., and Swaby, R. J. (1940).—Oxidation of manganous compounds by microorganisms in the soil. *Soil Sci.* **49**: 163–9.

McLachlan, J. D. (1941).—Manganese deficiency in soils and crops. 1. Control in oats by spraying; studies of the role of microorganisms. *Sci. Agric.* **22**: 201–07.

Mann, P. J. G., and Quastel, J. H. (1946).—Manganese metabolism in soils. *Nature* **158**: 154–6.

Martell, A. E., and Calvin, M. (1952).—"Chemistry of the Metal Chelate Compounds." p. 613. (Prentice-Hall Inc.: New York.)

Söhngen, N. L. (1914).—Umwandlungen von Manganverbindungen unter Einfluss mikrobiologischer Prozesse. *Zbl. Bakt.* **40**: 545–54.

Starkey, R. L. (1955).—Relations of micronutrients to development of microorganisms. *Soil Sci.* **79**: 1–14.

Thiele, G. A. (1925).—Manganese precipitation by microorganisms. *Econ. Geol.* **20**: 301–10.

Timonin, M. I. (1946).—Microflora of the rhizosphere in relation to the manganese deficiency disease of oats. *Proc. Amer. Soc. Soil Sci.* **11**: 284–94.

Timonin, M. I. (1950*a*).—Soil microflora in relation to manganese deficiency. *Sci. Agric.* **30**: 324–5.

Timonin, M. I. (1950*b*).—Soil microflora and manganese deficiency. Trans. 4th Int. Congr. Soil Sci. Amst. Vol. 3. pp. 97–9.

Waksman, S. A. (1940).—Soil microbiology. *Ann. Rev. Biochem.* **9**: 509–22.

EXPLANATION OF PLATES 1 AND 2

PLATE 1

Fig. 1.—Photomicrograph showing deposition of manganese oxide in colonies of *Corynebacterium* strain A which have been overgrown by the spreading colony of *Chromobacterium* sp. × 10.

Fig. 2.—Photomicrograph showing sector of *Corynebacterium* strain B. × 10.

Fig. 3.—Photomicrograph of *Corynebacterium* strain A and *Corynebacterium* strain B isolated from sector shown in Figure 2. × 10.

Fig. 4.—Photomicrograph showing reversion of *Corynebacterium* strain B to *Corynebacterium* strain A. × 20.

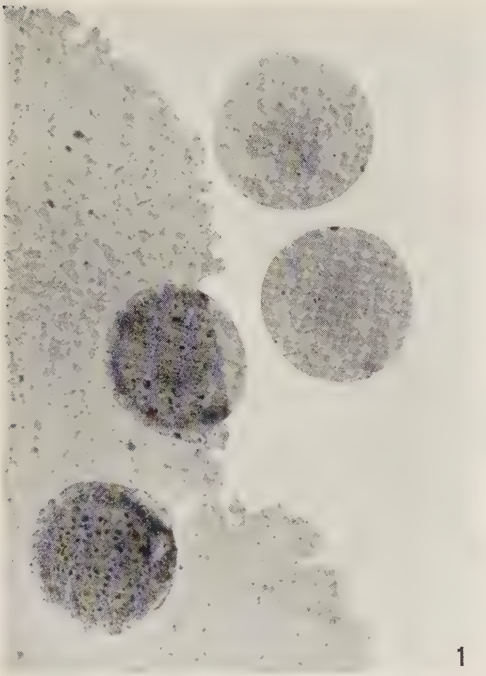
PLATE 2

Fig. 1.—Photomicrograph of the aggregates in the oxidation product showing the intimate mixture of oxide and bacterial cells. × 2125.

Fig. 2.—Photomicrograph showing the aggregates which clump together to form the sponge-like oxidation product. × 1125.

Fig. 3.—Photomicrograph of the product of biological oxidation of manganese showing its sponge-like nature. × 30.

OXIDATION OF MANGANESE BY SOIL MICROORGANISMS



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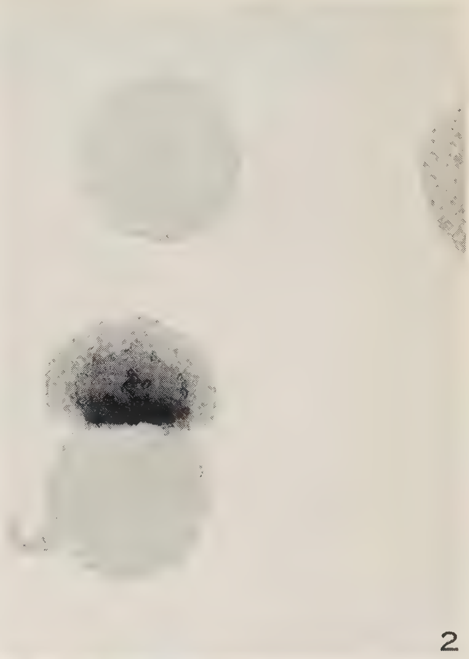

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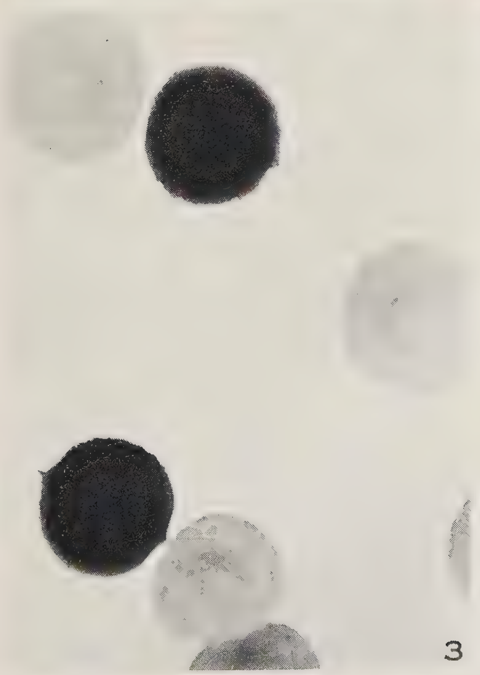

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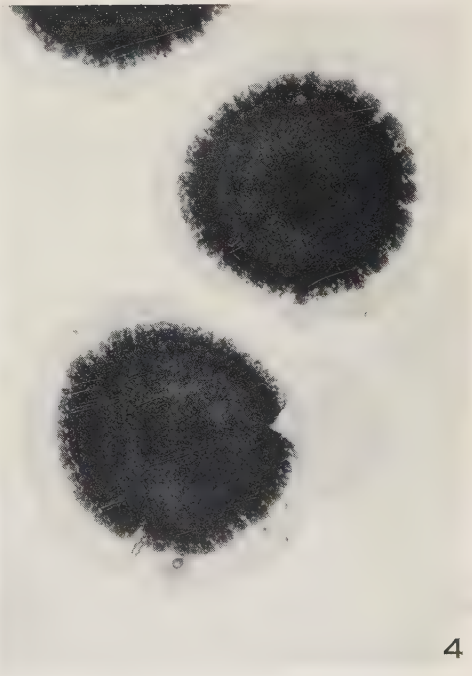

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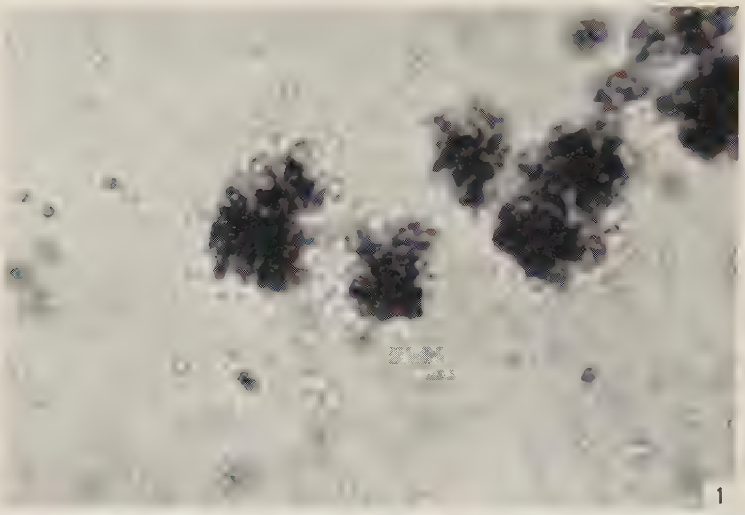

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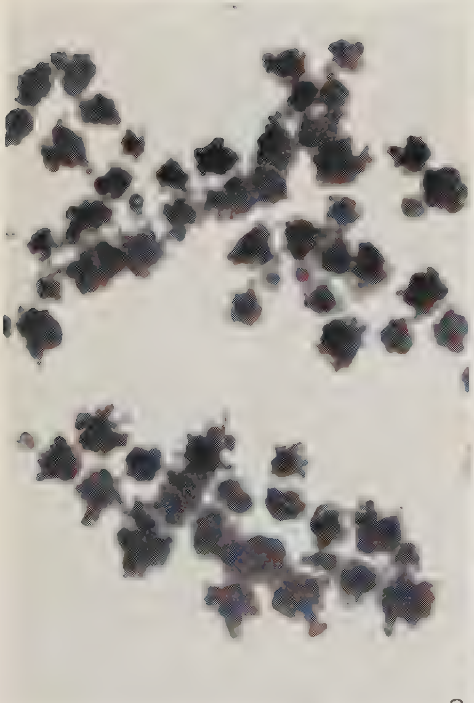

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THE METABOLISM OF ARGININE IN *SERRATIA MARCESCENS*

II. CARBAMYL–ADENOSINE DIPHOSPHATE PHOSPHOFERASE

By K. T. Glasziou*

[*Manuscript received November* 22, 1955]

Summary

Carbamyl–adenosine diphosphate (ADP) phosphoferase, an enzyme which catalyses the synthesis and breakdown of carbamyl phosphate (CAP), has been purified 43-fold and obtained free of carbamyl phosphate phosphatase activity. ADP, but not adenosine monophosphate, has been shown to be a substrate for the enzyme. Magnesium or manganous ions are required for activity. Inhibition by heavy metal cations and *p*-chloromercuribenzoate indicate that a sulphydryl group is involved in catalysis.

The mechanisms of carbamyl-ADP phosphoferase action and some aspects of citrulline metabolism are discussed.

I. Introduction

In a study of *Serratia marcescens* (Glasziou 1956), no urease, arginase, or arginine desmidase activity could be detected. Evidence was given that the conversion of citrulline to arginine proceeded via argininosuccinic acid. Phosphorolysis and arsenolysis of citrulline were demonstrated. Unlike bacteria such as *Streptococcus faecalis* and *Staphylococcus aureus*, the rate of phosphorolysis of citrulline was not increased by the addition of adenosine phosphates to the reaction mixture. In this respect the system in *S. marcescens* is similar to that found in mammalian liver (Krebs, Eggleston, and Knivett 1955).

Jones, Spector, and Lipmann (1955) showed carbamyl phosphate (CAP) to be an intermediate in the biosynthesis of citrulline from ornithine by extracts from *Strep. faecalis* and also from liver mitochondria. CAP was dissimilated in the presence of acetone-dried cells of *S. marcescens* under conditions which give rise to phosphorolysis of citrulline in bacterial and animal systems. The reaction was followed by measurement of CO_2 output, and a marked stimulation of the reaction rate was observed in the presence of adenosine diphosphate (ADP). Thus it appeared that there were two pathways for the breakdown of CAP, one a hydrolysis, and the other a transfer of energy-rich phosphate to ADP. This paper reports the preparation of carbamyl–ADP phosphoferase from *S. marcescens* free of detectable phosphatase activity, and describes some of the properties of the enzyme.

II. Materials

Acetone-dried cells of an arginine-requiring mutant from a laboratory strain of *S. marcescens* were used as the starting material for all the experiments reported. This mutant grows on arginine or citrulline but not ornithine or CAP. Arsenolysis of citrulline does not occur so that carbamyl-ornithine kinase is inactive. The methods of preparation of acetone-dried cells, CAP, and *N*-acetyl glutamic acid were described in Part I of this series (Glasziou 1956). Adenosine-5-monophosphate (AMP), ADP, and adenosine triphosphate (ATP) were commercial preparations (Pabst Laboratories Ltd.).

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III. METHODS

(*a*) *Cell-free Extracts*

Acetone-dried cells were extracted by grinding in a mortar in either buffer solution or distilled water. The suspension was kept at 0–1°C and stirred at intervals. The time and medium for extraction were varied as required. In some experiments the extraction was made with distilled water and with dialysis at 0–1°C. In such cases it was necessary to add buffer before centrigufation to facilitate packing of the residues. Centrifugation was carried out in an International refrigerated centrifuge, model PR2, at 25,000 *g* for 10–15 min. The clear supernatant was withdrawn and kept at 0–1°C until required.

(*b*) *Preparation of Carbamyl–ADP Phosphoferase*

Acetone-dried cells (400 mg) were extracted with 10 ml of 0·25M Sorensen's phosphate buffer, pH 6·1, for 2 hr at 0–1°C. After centrifugation, aliquots of the cell-free extract were removed for protein determination and for enzyme assays. To 6·5 ml of the extract an equal volume of sat. ammonium sulphate was added. The precipitated protein was collected by centrifugation at 25,000 *g* for 15 min, the supernatant being discarded. The precipitate was dissolved in 3 ml of 0·01M phosphate buffer, pH 6·4. The temperature throughout the ammonium sulphate fractionation was maintained at 0–1°C.

To the 3 ml of enzyme solution, absolute ethanol at 0°C was added dropwise and with constant stirring. The temperature during this step was between 0 and 3°C. At lower temperatures (ethanol at −10°C) carbamyl phosphate phosphatase (CAP-ase) activity was preserved, and contaminated the carbamyl–ADP phosphoferase fraction. Fractions were collected at concentrations of 55 per cent. (v/v) ethanol and from 55–62 and 62–67 per cent. (v/v) ethanol. The precipitated material of each fraction was collected by centrifugation and taken up in 5 ml of 0·25M phosphate buffer, pH 6·1. Of these three fractions, the major part of the enzyme activity was in the 62–67 per cent. fraction. The data for this fractionation are given in Table 1. The enzyme assays and protein determinations were completed within 3 hr of commencing centrifugation of the suspension.

From this procedure a standardized preparation was developed in which 500 mg cells were extracted overnight in 10 ml of buffer. The precipitate from half saturation with ammonium sulphate was taken up in 3 ml of 0·01M buffer as before and 5 ml ethanol added. The precipitate was discarded and a further 1·5 ml ethanol added to the supernatant. The precipitate from this step was collected and taken up in 5 ml buffer or water as required. This solution is subsequently referred to as the carbamyl–ADP phosphoferase preparation.

(*c*) *Enzyme Assay*

The two enzymes were assayed by the manometric measurement of CO_2 evolution using Warburg manometers. The enzyme unit of CAP-ase activity was defined as that amount of enzyme which catalysed the formation of 1 μl CO_2 per min in a system which contained 15 μmoles CAP, 20 μmoles $MgSO_4$, and 125 μmoles phosphate buffer, pH 6·1, in a total vol. of 3·3 ml at 30°C and in an initial gas phase of nitrogen. The unit of carbamyl–ADP phosphoferase activity was similarly defined except that 20 μmoles ADP were included in the reaction mixture. In both cases corrections were made for the spontaneous breakdown of CAP. The rates were measured under conditions which closely approximated zero order reactions.

TABLE 1

SEPARATION OF CARBAMYL–ADP PHOSPHOFERASE (PHOSPHOFERASE) FROM CARBAMYL PHOSPHATE PHOSPHATASE (CAP-ASE)

Step	Volume (ml)	Enzyme Activity (units/ml)		Protein (mg/ml)	Ratio of Absorption (280/260 mμ)	Specific Activity		Percentage Yield of Phosphoferase	Purification
		Phosphoferase	CAP-ase			Phosphoferase	CAP-ase		
Original extract	6·5	8·0	0·4	6·2	0·84	1·3	0·065	100	—
Ethanol fraction* (62–67%)	5·0	4·1	—	0·073	0·93	56	—	40	43×

* See text for fractionation procedure.

(*d*) *Determinations*

Methods for deproteinization, measurement of CAP, and orthophosphate have been cited previously (Glasziou 1956). Ammonia was determined by a Conway method modified from the method described by Braganca, Quastel, and Schuchen (1954). Protein was determined by measurement of the optical density of the solutions at 260 and 280 mμ (Hilger Uvispek) and applying the formula derived by Kalckar (1947). CO_2 was measured using standard Warburg manometric technique.

IV. RESULTS

(*a*) *The Separation of Carbamyl–ADP Phosphoferase from CAP-ase*

The two activities towards CAP found in acetone-dried cells of *S. marcescens* could conceivably be catalysed by one enzyme. Preliminary experiments, in which the ratios carbamyl–ADP phosphoferase/CAP-ase were measured using a variety of extraction conditions, gave values ranging from 2·5 to 24. Fractionation procedures resulted in the preparation of carbamyl–ADP phosphoferase free of CAP-ase activity indicating that two enzymes are involved for the reactions catalysed (Table 1).

Recently Marshall, Hall, and Cohen (1955) showed that compound *X*, an intermediate in citrulline synthesis by rat liver preparations (Grisolia and Cohen 1952), was identical with CAP. The enzyme reported by Grisolia and Marshall (1954) which split compound *X* was presumably a CAP-ase.

(*b*) *The Stoichiometry of the Reaction Catalysed by Carbamyl–ADP Phosphoferase*

The results presented in Table 2 show that there is a stoichiometric relationship between the amount of CO_2, NH_3, and ATP formed during decomposition of CAP in the presence of a cell-free extract from *S. marcescens*.

TABLE 2

THE FORMATION OF ADENOSINE TRIPHOSPHATE, CO_2, AND NH_3, FROM CARBAMYL PHOSPHATE (CAP)

Extract (1·7 ml) buffered with 85 μmoles succinate buffer pH 6·1, 20 μmoles $MgSO_4$, 9·6 μmoles CAP (which contained 0·3 μmole NH_3), 15·5 μmoles ADP; total vol. 3·3 ml. System incubated in an atmosphere of N_2 at 30°C

Experiment	Treatment	CO_2 Formed (μmoles)	Labile Phosphorus* (μmoles)	Increase in Labile Phosphorus (μmoles)	NH_3 Formed (μmoles)
1	Stopped at zero time	—	15·5	—	
2	Stopped at zero time	—	15·5	—	
3	Incubated at 30°C	6·1	21·4	5·9	
4	Incubated at 30°C	6·3	21·5	6·0	
5	Incubated at 30°C	5·4			5·7
6	Incubated at 30°C	6·0			5·5

* That amount of orthophosphate hydrolysed in 8 min at 100°C in the presence of N HCl. The figures quoted are corrected for inorganic and CAP orthophosphate.

(*c*) *The Synthesis of CAP from ATP, CO_2, and NH_3*

The synthesis of CAP from ATP, CO_2, and NH_3 was demonstrated by Jones *et al.* (1955) using extracts from *Strep. faecalis* or liver mitochondria. This synthesis had previously been demonstrated by Grisolia and Cohen (1952) although the reaction product was not identified. The synthesis of CAP in the presence of carbamyl–ADP phosphoferase from *S. marcescens* proved difficult to demonstrate due to the increase in orthophosphate, which was not diminished by potassium fluoride (Table 3). The addition of *N*-acetyl glutamic acid did not increase the amount of CAP formed as it does in the rat liver system reported by Marshall *et al.* (1955).

(*d*) *The Effect of Adenosine Phosphates on the Breakdown of CAP*

No conclusive evidence has been presented to show which of the adenosine phosphates accepts the energy-rich phosphate group produced during the phosphorolysis of citrulline. Oginsky and Gehrig (1953) using acetone-dried cells of *Strep. faecalis* found that AMP was the best phosphate acceptor under the conditions of their experiments. The addition of Mg^{++} stimulated both ATP breakdown to yield AMP (apyrase was present in the system) and the acceptance of an energy-rich phosphate group by AMP.

TABLE 3

THE SYNTHESIS OF CARBAMYL PHOSPHATE FROM ADENOSINE TRIPHOSPHATE (ATP), CO_2, AND NH_3

The complete system contained 1·0 ml carbamyl–ADP phosphoferase preparation containing 500 μmoles tris(hydroxymethyl)aminomethane buffer pH 7·8, 20 μmoles $MgSO_4$, 20 μmoles ATP, 100 μmoles $(NH_4)_2CO_3$; final vol. 3·0 ml. System incubated for 60 min at 30°C

System	Inorganic Phosphate (μmoles)	Unstable Phosphate* (μmoles)
No enzyme	1·94	0·18
No ATP	0·73	0·06
Complete	4·25	0·6
Complete + 20 μmoles *N*-acetyl glutamate	3·85	0·5

* That amount of orthophosphate hydrolysed in 1 min at 100°C in the presence of 0·01N HCl. This value defines CAP (Jones *et al.* 1955).

The breakdown of CAP in the presence of partially purified carbamyl–ADP phosphoferase from *S. marcescens* takes place with ADP but not AMP or ATP as phosphate acceptor (Table 4).

TABLE 4

THE EFFECT OF ADENOSINE PHOSPHATES ON THE BREAKDOWN OF CARBAMYL PHOSPHATE

The complete system contained 0·5 ml carbamyl–ADP phosphoferase preparation buffered with 125 μmoles phosphate buffer pH 6·1, 20 μmoles $MgSO_4$, 20 μmoles adenosine phosphate (AMP, ADP, or ATP), 10 μmoles CAP; total vol. 3·3 ml. System incubated in an atmosphere of N_2 at 30°C

System	CO_2 Produced (μl)		
	10 Min	30 Min	60 Min
No enzyme	2	7	12
No adenosine phosphate	2	6	12
Complete (AMP)	2	7	12
Complete (ADP)	55	123	143
Complete (ATP)	6	12	16

(e) *The Effect of* N*-Acetyl Glutamic Acid on Carbamyl–ADP Phosphoferase*

The catalytic effect of derivatives of glumatic acid on the synthesis of compound *X* (CAP) has been well established for liver preparations. Grisolia and Cohen (1953) showed that *N*-acetyl glutamate, *N*-chloroacetyl glutamate, carbamyl glutamate, and *N*-formyl glutamate were effective in that order. Krebs *et al.* (1955) found that the phosphorolysis of citrulline in the presence of *Eschericia coli* and liver enzymes was accelerated by carbamyl glutamate, carbamyl glutamine, acetyl glutamate, and carbamyl alanine. The concentrations of amino acid derivatives used by Krebs *et al.* (1955) were much greater than those required for catalysis of CAP formation. The most likely role of these substances appears to be as cofactors of carbamyl–ADP phosphoferase or as precursors of a cofactor.

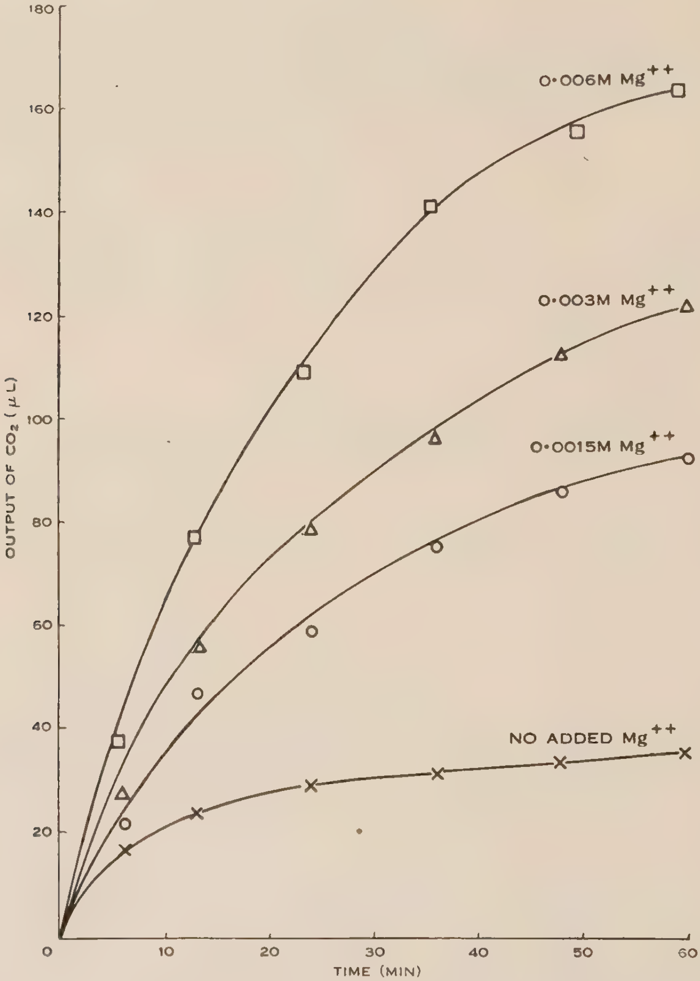

Fig. 1.—The effect of Mg++ on the breakdown of CAP. In addition to $MgSO_4$, the reaction mixtures contained 0·5 ml carbamyl–ADP phosphoferase preparation buffered with 125 μmoles phosphate buffer pH 6·1, 20 μmoles ADP, 10 μmoles CAP, total vol. 3·3 ml. System incubated in an atmosphere of N_2 at 30°C.

Attempts to demonstrate an effect by *N*-acetyl glutamate, the most effective of these catalysts, on carbamyl–ADP phosphoferase prepared from *S. marcescens* have not met with any success either on the synthesis or breakdown of CAP. Dialysis of the enzyme in dialysis tubing which had been soaked for 24 hr in distilled water resulted in partial or complete inactivation. The activity was not restored by *N*-acetyl glutamate but was restored by either cysteine, glutathione, or boiled liver extract. Further attempts are being made to split the enzyme into an apoenzyme and a prosthetic group.

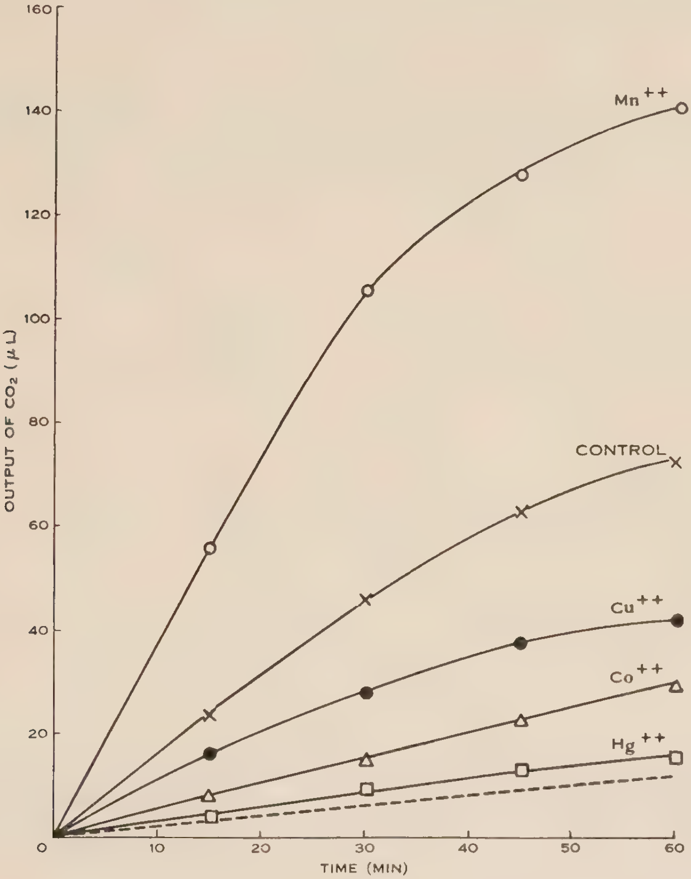

Fig. 2.—The effect of divalent cations on the breakdown of CAP. The reaction mixtures contained 0·5 ml carbamyl–ADP phosphoferase preparation buffered with 125 μmoles phosphate buffer pH 6·1, 20 μmoles ADP, 10 μmoles CAP, 5 μmoles $MgSO_4$, total vol. 3·3 ml. System incubated in an atmosphere of N_2 at 30°C. Added cations were at a final concn. of 0·006M. The dotted line gives the spontaneous breakdown of CAP under the conditions of the experiment.

(*f*) *The Effect of Divalent Cations and* p-*Chloromercuribenzoate on Carbamyl–ADP Phosphoferase*

Mg^{++} is required for the synthesis of CAP (compound *X*) by liver preparations (Grisolia and Cohen 1952; Jones *et al.* 1955) or by extracts of *Strep. faecalis* (Jones *et al.* 1955). Oginsky and Gehrig (1953) reported a requirement for Mg^{++} or Mn^{++} in the phosphorolysis of citrulline by *Strep. faecalis*, and observed inhibition by Cu^{++} and Hg^{++}. The inhibition by Hg^{++} was reversed by glutathione and British anti-lewisite (1, 3-dimercaptopropanol). The reaction was also inhibited by *p*-chloromercuribenzoate and this effect could be reversed by glutathione. This suggests that an –SH group is functional in one of the enzymes of the "citrullinase" system.

Table 5

THE EFFECT OF *p*-CHLOROMERCURIBENZOATE ON CARBAMYL–ADP PHOSPHOFERASE

The complete system contained 0·5 ml carbamyl–ADP phosphoferase preparation buffered with 125 μmoles phosphate buffer pH 6·1, 30 μmoles $MgSO_4$, 10 μmoles CAP, 20 μmoles ADP; total vol. 3·3 ml. System incubated in an atmosphere of N_2 at 30°C

System +		Output of CO_2 (μl)		
p-Chloromercuribenzoate (final concn.)	Glutathione (final concn.)	10 Min	35 Min	78 Min
—	—	40	78	109
—	1×10^{-3}M	35	69	102
2×10^{-5}M	—	17	21	31
2×10^{-5}M	1×10^{-3}M	32	66	98
2×10^{-6}M	—	16	22	35
2×10^{-6}M	1×10^{-3}M	37	72	103
2×10^{-7}M	—	38	70	104
2×10^{-7}M	1×10^{-3}M	34	68	100

The necessity for either Mg^{++} or Mn^{++} in the breakdown of CAP by carbamyl–ADP phosphoferase from *S. marcescens* is shown in Figures 1 and 2. Inhibition by Hg^{++}, Co^{++}, and Cu^{++} is shown in Figure 2.

The effect of *p*-chloromercuribenzoate was tested (Table 5). The inhibition by this substance was reversed by glutathione. These data suggest that the –SH group is important for the catalytic activity of the enzyme.

V. Discussion

The proposal that the reaction catalysed by carbamyl-ornithine kinase is rate-limiting in the phosphorolysis of citrulline was put forward to explain the lack of stimulation of the reaction rate by added adenosine phosphates in *S. marcescens* and liver preparations (Glasziou 1956). The marked stimulation by phosphate acceptors in bacteria such as *Strep. faecalis* and *Staph. aureus* could be due to a greater content of carbamyl-ornithine kinase. If this explanation is correct then gross metabolic differences exhibited by different organisms are due to alteration in relative enzyme content rather than a change in enzyme pattern. It has been shown that ADP and not AMP will accept energy-rich phosphate from CAP in the presence of carbamyl–ADP phosphoferase from *S. marcescens*. Purification of the corresponding enzyme from *Strep. faecalis* may show that AMP is the acceptor in this system as claimed by Oginsky and Gehrig (1953). However, if this were so it would not invalidate the argument that the carbamyl-ornithine kinase reaction controls the overall properties of the citrullinase system.

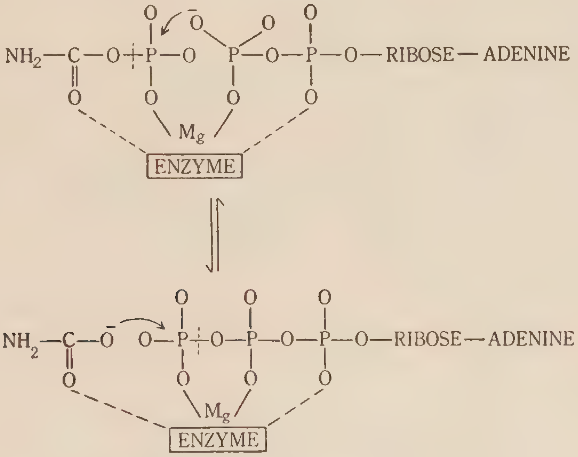

Fig. 3.—The possible role of metal ions in the reaction catalysed by carbamyl–ADP phosphoferase.

The role of glutamate derivatives remains obscure. The best hypothesis that can be proposed at present appears to be that a glutamic acid derivative with a *N*-carbonyl grouping is functional in the active centre of carbamyl–ADP phosphoferase, and that the cofactor is readily dissociated from the liver enzyme but not from the bacterial enzyme.

The transfer of energy-rich phosphate to ADP during phosphorolysis of citrulline may involve the participation of an enzyme-phosphoryl linkage (Krebs *et al.* 1955; Glasziou 1956). Alternatively, the mechanism of action proposed for pyruvate–ADP phosphoferase by Boyer and Harrison (1954) would equally well explain the reaction catalysed by carbamyl–ADP phosphoferase and at the same time account for the incorporation of ^{18}O from orthophosphate into CO_2 during phosphorolysis of citrulline (Stulberg and Boyer 1954). The participation of Mg^{++} or Mn^{++} in the forward and reverse reaction is best explained by assuming complex formation with the enzyme and both substrates, the high charge density of the metal ion mediating an electronic shift and thereby increasing the residual positive charge on the terminal phosphorus atom of ATP or CAP (Fig. 3). In the reaction mechanism postulated the metal ion is also functional in binding ATP to the enzyme. Cohn (1953), using the paramagnetic absorption resonance technique, has demonstrated that Mn^{++} forms chelate compounds with ATP and other organic phosphates.

VI. Acknowledgments

I wish to thank the Research Committee, University of Sydney, for the receipt of a research grant.

VII. References

Boyer, P. D., and Harrison, W. W. (1954).—"A Symposium on the Mechanism of Enzyme Action." (Ed. W. D. McElroy, and B. Glass.) (The John Hopkins Press: Baltimore.)
Braganca, B. M., Quastel, J. A., and Schucher, R. (1954).—*Arch. Biochem. Biophys.* **52**: 18.
Cohn, M. (1953).—*J. Biol. Chem.* **201**: 735.
Glasziou, K. T. (1956).—*Aust. J. Biol. Sci.* **9**: 117.
Grisolia, S., and Cohen, P. P. (1952).—*J. Biol. Chem.* **198**: 561.
Grisolia, S., and Marshall, R. O. (1954).—*Biochem. Biophys. Acta* **14**: 446.
Jones, M. E., Spector, L., and Lipmann, F. (1955).—*J. Amer. Chem. Soc.* **77**: 819.
Kalckar, H. (1947).—*J. Biol. Chem.* **167**: 461.
Krebs, H. A., Eggleston, L. F., and Knivett, V. A. (1955).—*Biochem. J.* **59**: 185.
Marshall, R. O., Hall, L. M., and Cohen, P. P. (1955).—*Biochem. Biophys. Acta* **17**: 279.
Oginsky, E. L., and Gehrig, R. H. (1953).—*J. Biol. Chem.* **204**: 721.
Stulberg, M. P., and Boyer, P. D. (1954).—*J. Amer. Chem. Soc.* **76**: 5569.

LEUCOANTHOCYANINS AS THE POSSIBLE PRECURSORS OF EXTRACTIVES IN WOODY TISSUES

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Summary

Leucoanthocyanins in plant tissues have been estimated by measuring the anthocyanidins produced after heating with *iso*propanol–hydrochloric acid in a closed tube. Various tissues of *Eucalyptus sieberiana* F. Muell., *E. obliqua* L'Herit., *E. regnans* F. Muell., and *E. maculata* Hook. were studied by this method. The amount of leucoanthocyanins was observed to decrease with increasing degree of maturity of the different leaves on a stem. Most of the leucoanthocyanins appeared to be in the colourless leaf tips. The amount of leucoanthocyanin in the bark decreased during the growing season, and the amount in the cambium decreased rapidly once differentiation of the tissues began. The sapwood of *E. sieberiana* contained about half that in the heartwood. Similar results were found in transverse discs from logs of *E. marginata* Sm. which contained also an outer heartwood zone. The extraneous materials in this latter zone were much more water soluble, although the alkali solubles of both the inner and outer heartwood zones were the same. The acetone solubles in *E. marginata* sapwood were largely leucoanthocyanins.

Chromatographic examination with different solvents of the alcohol extracts from different tissues of *E. sieberiana* showed that the anthocyanin in the leaves was different from that in the bark. The amount of flavone glycosides decreased rapidly in samples taken progressively further away from the leaves. On the other hand, a mauve fluorescent component increased. Examination of the reaction products obtained after heating with *iso*propanol–hydrochloric acid showed that leucoanthocyanins which yielded delphinidin and cyanidin were present in all tissues except the cambium which yielded only cyanidin; the kino also yielded only cyanidin.

The view is expressed that the leucoanthocyanins in the xylem originate from the leaves and that they are the immediate precursors of bark and heartwood extractives. Evidence is given of the ease with which leucoanthocyanins are converted to other flavonoids.

I. Introduction

Leucoanthocyanins or leucoanthocyanidins are detected by heating the sample with aqueous or alcoholic hydrochloric acid when a red colour, largely due to anthocyanidin, is produced. They have been shown to be widespread in plant tissues (e.g. Robinson and Robinson 1933; Robinson 1937; Lawrence *et al.* 1939; Bate-Smith 1954; and Bate-Smith and Lerner 1954). Positive reactions to the above test have been reported for many species (Isenberg and Buchanan 1945; Stearns and Hartley 1952; Towers and Gibbs 1953) although leucoanthocyanins were not recognized as the responsible substances.

Bate-Smith (1953) showed that they occur much more commonly in the tissues of the woody plants than in those of the herbaceous plants. Large amounts have been shown to be present in the bark of certain eucalypts (Hillis 1954). Robinson and Robinson (1933) reported that they are found in larger amounts in the heartwood than in the sapwood and they (1935) isolated the first leucoanthocyanidin from the heartwood of *Peltogyne pubescens* Bth. Recently Pigman *et al.* (1953) isolated a leuco-

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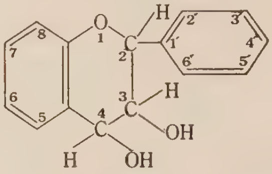
anthocyanin from the inner bark of *Picea mariana* B.S.P. and King and Bottomley (1954) isolated a leucoanthocyanidin from the heartwood of *Acacia melanoxylon* R. Br.

Bate-Smith and Lerner (1954) considered that the production of leucoanthocyanins is part of a primitive metabolic pattern associated with, but not essential to, a woody habit of growth. They also discussed the relationship of these substances to lignin and tannin.

Whilst it is agreed that under special circumstances they may be the precursors of anthocyanins (Robinson and Robinson 1939; Rutzler 1939), the existing contention is that they are derived, at least in part, from a common pigment precursor (Scott-Moncrieff 1939).

Recent work (e.g. Bauer, Birch, and Hillis 1954; Swain 1954) has shown that the leucoanthocyanidins have the basic structure:



This is the cyclic form of the compound C_6–CH(OH)–CH(OH)–CH(OH)–C_6 which Robinson (1936) postulated arose from sugars and was the precursor of the flavonoids and the anthocyanidins. Consequently leucoanthocyanidins could be one of the first forms of polyphenolic compounds synthesized in the plant and could be the precursor of flavonoids, anthocyanidins, and tannins found in various woody tissues.

Szent-Györgyi (1937) and Reichel and Burkhart (1938) have considered that certain flavones play an important part as components of the oxidation-reduction systems of the plant. Leucoanthocyanidins can be theoretically transformed to flavonoids and anthocyanidins by dehydrogenation, hydrogenation, dehydration, and pyran-ring opening. The ubiquitous leucoanthocyanins could play, then, an important role in the respiration of plant tissues, and the more stable compounds usually isolated from these tissues being the product of the respiration therein.

This present work reports the examination of tissues of certain species of eucalypts. Leucoanthocyanins were estimated to ascertain whether large amounts are found in those tissues where sugars are formed or accumulate, which would be expected if leucoanthocyanins are an early stage in the path of sugars to tannin. Also a chromatographic examination of extracts from these tissues was made to gain an idea of the nature of the accompanying components and whether there was any relationship between them.

II. Material Used

(a) *Leaves*

Leaves from branchlets of the following species were examined: *Eucalyptus sieberiana* F. Muell. (silvertop ash), *E. obliqua* L'Herit. (messmate), *E. capitellata* Sm. (brown stringybark), *E. regnans* F. Muell. (mountain ash), *E. elaeophora* F. Muell. (long leaved box), and *E. maculata* Hook. (spotted gum). They were obtained at different periods during the unusually wet and late spring of 1954.

A notable feature of the spring growth of *E. sieberiana* is the bright red colour of the leaves. Other eucalypts show a red or red-brown colour but not to the same intensity. At the height of spring growth a typical stem of *E. sieberiana* will carry ten or more red leaves with the third and fourth from the apex being the most intensely coloured. The number of red leaves decreases as the season progresses.

In the youngest completely unfolded leaf, the extreme tip (one-fifth of the length) is almost devoid of any colour. Adjacent to it is a pink-red area (one-third of the length) and the rest of the leaf is a very pale pink; the marginal vein is distinctly pink and the midrib greenish yellow. With the exception of the tips, which are pale green, the whole of the third and fourth unfolded leaves are red in colour, but the veins appear to be less intensely red. The sixth to eighth leaves, still with pale green tips, show signs of green on both sides of the midrib in the central portion; this area increases in older leaves until only the marginal vein is red and finally this too becomes green. When green first appears in the leaf the veins become redder than formerly.

(*b*) *Stems of Branchlets*

The external portion of the new season's growth of *E. sieberiana* is reddish yellow near the apex, becoming brilliant red further along, the colour reaching a maximum at about the position where the fifth or sixth leaf is attached. This colour is present in the next 2–3 ft or more but decreases in intensity. The stems are still red some distance from where the leaves have become completely green. The external layer of the under-portions of the branchlets takes on a green colour at a distance much closer to the apex than does the upper surfaces.

A similar pattern of colour changes was observed with other species but it was less marked.

(*c*) *Cambial Zone*

The cambial zone was gently removed with a "Perspex" scraper immediately after the phloem and bark was lifted. When it was removed from a log of *E. sieberiana* collected on November 15, 1954 the colourless material became distinctly pink in about a minute, becoming increasingly brown after 5 min. This transient pink colour did not appear when samples were collected later in the season.

(*d*) *Bark Samples*

Sections of the tree trunk were collected at the same time as the leaves and were stored at 5°C. The samples of bark were taken from different positions on the tree at a distance between 0·05–0·10 in. from the cambial zone.

(*e*) *Wood Samples*

The samples of sapwood and heartwood of *E. sieberiana* were taken from the transverse sections referred to in Section II (*d*). As is the case with many tree species, the change of sapwood to heartwood was abrupt, with a change in the colour of the wood and an increase in the amount of extraneous materials present in the cavities of the various cells particularly the rays and vertical parenchyma.

Occasionally in log cross sections of jarrah (*E. marginata* Sm.) a clear red coloured outer heartwood zone contrasts with the inner heartwood possessing the more usual brown-red colour. Zones up to 1·25 in. wide in a 12-in. diameter section have been observed, and also a lenticular shaped zone (0·5 × 3 in.) was seen in a cross section in which the normal abrupt change of sapwood to heartwood occurred elsewhere. The transverse discs of *E. marginata* were collected from mature trees (more than 100 years old) and from regrowth trees (about 50 years old) grown in Western Australia.

(*f*) *Kino*

Liquid kino was collected from the heartwood of *E. sieberiana* (log, December 7, 1954) and 1·5 in. from the boundary with the sapwood. Solid kino which was fresh in appearance was collected in a crevice in the bark from another tree of this species.

III. Experimental Procedure

(*a*) *Estimation of Leucoanthocyanin*

(i) *Method.*—A development of the method introduced by Pigman *et al.* (1953) and further applied by Hillis (1954) was used as follows: immediately after preparation, the sample was weighed and heated with 10 ml of *iso*propanol: 3·0N HCl (5: 1) in a tube (15 × 1 cm) equipped with a ground-glass stopper which was held in place with spring clips. The tube was heated in boiling water for exactly 40 min with occasional tipping to mix the contents, and then cooled to room temperature in a beaker of cold water. The filtrate from the tube was quantitatively diluted with *iso*propanol until the transmission was between 20–30 per cent., then the optical density was determined accurately between 520 and 565 mμ with a Unicam spectrophotometer model S.P. 500. Chromatographic examination (see Section III(*b*)) indicated that delphinidin was the main anthocyanidin produced and the maximum absorption (550–555 mμ) supported this (Bate-Smith 1954). The leucoanthocyanin was calculated from the anthocyanidin produced on the provisional assumptions that the conversion was complete and that delphinidin was the only anthocyanidin produced. The results were corrected for the effect of dilution, then calculated to dry weight.

Table 1

DISTRIBUTION OF LEUCOANTHOCYANIN IN A EUCALYPTUS OBLIQUA LEAF

Section	1	2	3	4	5	6
Distribution (%)	1·67	2·67	5·26	3·99	3·85	3·04

The leaves were analysed within a few hours of collection, as it was found that the leucoanthocyanin decreased on storage, particularly in the pale tips of the leaves which quickly shrivelled and became discoloured. The *E. obliqua* leaf (Table 1; Fig. 1) had been stored for 2 days when the amount of leucoanthocyanin was reduced, mostly in the tip.

(ii) *Effect of Natural Red Colour.*—No attempt was made to correct the results for the natural red colour of the leaves as it was found that it did not make any real difference. This colour was reddest in the third unfolded leaves from the apex and was found to be 5·4 and 8·1 per cent. of the total red colour produced after heating with *iso*propanol-hydrochloric acid.

(iii) *Effect of Background Colour.*—In addition to the leucoanthocyanins, the *iso*propanol in the reagent removes many other substances, and of these, chlorophyll appears to be the principal source of the background colour. This colour interferes somewhat with the estimation of anthocyanidin. Although there is much less interference when aqueous hydrochloric acid is employed for the conversion to anthocyanidin, the author has found this reagent to be less sensitive and less suitable for quantitative work. Several attempts were made to estimate the background colour but, as it was not possible to do so accurately, no corrections have been made.

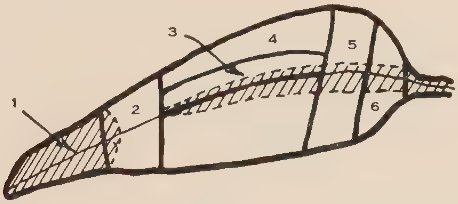


Fig. 1.—Sections taken from an *E. obliqua* leaf. Approximately natural size. Unshaded portions red.

(iv) *Sampling.*—In order to ascertain whether leucoanthocyanins are distributed uniformly, an *E. obliqua* leaf which was mainly red was sectioned as shown in Figure 1. The leucoanthocyanin content of the portions is shown in Table 1. Obviously, the analysis of a random section of a leaf would not represent that of the whole leaf. The present procedure has been to take all of the small leaves (less than 2 in. long) and one-half of the larger ones after slitting them along the midrib.

To ascertain the effect of increasing the ratio of sample to reagent beyond the optimum limits, a complete leaf was finally minced, then thoroughly mixed, and various amounts taken and analysed as in Section III(*a*)(i), the whole procedure being completed as quickly as possible. The reaction products were diluted so that the suitable transmission was obtained. It was found that when the ratio of sample to reagent was increased there was a linear decrease in the anthocyanidin produced, e.g. a 10 per cent. decrease when a 4 times dilution was needed to obtain a suitable transmission, 20 per cent. for a 7·5 times dilution, and 40 per cent. for a 15 times dilution. Almost all the dilutions needed in the routine analyses of leaves were less than a 10 times dilution, and all results have been corrected for dilution effects. Standard conditions of technique were observed in all the leucoanthocyanin estimations and the results are reproducible to within 5 per cent.

(*b*) *Paper Chromatography of Extracts of Tissues*

The solvent-ascent method was used (at 22°C) in the normal manner. The ethanol extracts and the *iso*propanol-hydrochloric acid reaction products were

Table 2

RATIO OF DELPHINIDIN TO CYANIDIN IN DIFFERENT TISSUES AFTER *iso*PROPANOL–HYDROCHLORIC ACID TREATMENT

Solvent	Location									
	Red Leaves	Green Leaves	Petiole of 3rd Leaf	Red Outer Layer of Stem		Green Outer Layer 12. in from Petiole of 3rd Leaf	Bark	Cambium	Heart-wood	Kino
				2 in. from Petiole of 3rd Leaf	12 in. from Petiole of 3rd Leaf					
$CH_3COOH : HCl : H_2O$ (30 : 3 : 10 v/v)	4 : 1	3 : 1	1 : 2	2 : 1	2 : 1	3 : 1	4 : 1	Delphinidin only	4 : 1	Delphinidin only
$CH_3COOH : HCl : H_2O$ (10 : 2 : 20 v/v)	1 : 2	1 : 1	1 : 4	1 : 1	2 : 1	3 : 1	4 : 1	—	4 : 1	8 : 1
Wavelength at max. absorption (mμ)	555	555	550	550	550	555	550	550	555	555

prepared from similar amounts of tissue and kino samples using the same proportion of liquid to sample. The samples were repeatedly chromatographed and R_F values were within $\pm$ 0·02 R_F. Delphinidin, cyanidin, and quercetin were used as controls.

The resolving solvents used were (i) *n*-butanol: acetic acid: water (40: 10: 50 v/v); (ii) *n*-butanol: 2N HCl (1: 1 v/v); (iii) acetic acid: conc. HCl: water (30: 3: 10 v/v) (the "Forestal solvent" (Bate-Smith 1954)); (iv) acetic acid: conc. HCl: water (10: 2: 20 v/v) (Bottomley 1954); (v) phenol: 2N acetic acid+HCl (1: 1 v/v) (Hillis 1950).

The chromatograms were examined as soon as they were removed from the tanks in order to locate the anthocyan(id)ins, then, as soon as the sheets were air dry, were again examined under ultraviolet light before and after fuming with ammonia. The sheets were also sprayed with ammonia-silver nitrate or dipped in a freshly-mixed solution of ferric chloride and potassium ferricyanide (Barton, Evans, and Gardner 1952).

Delphinidin was usually present in the reaction products from the leucoanthocyanins in greater amounts than cyanidin but the ratio varied according to the location of the sample in the tree (see Table 2). The ratios obtained with the Forestal solvent (30: 3: 10 v/v) are probably the most reliable.

(*c*) *Wood Analysis*

In order to indicate the changes which take place when heartwood is formed in *E. marginata*, the hot water, alkali, and acetone soluble materials were determined. The first solvent removed low molecular weight carbohydrates or low molecular weight polyphenols or both, the second certain carbohydrates or polyphenolic substances or both, and the third mainly low molecular weight polyphenols. The methods used were as follows:

(i) *Hot Water Soluble Material.*—Two grams of accurately weighed sample (ground to pass a 20 mesh sieve) were heated with 300 ml of distilled water on a boiling water bath for 1 hr. The suspension was filtered hot through a coarse porosity alundum crucible (R.A. 98) and all the sawdust transferred to the crucible and washed thoroughly with 500 ml of boiling water. The extracted sawdust was dried under vacuum at 45°C to constant weight.

(ii) *Alkali Soluble Material.*—As in Section III(*c*)(i) but with 300 ml 0·1N NaOH instead of distilled water.

(iii) *Acetone Soluble Material.*—Ten grams of accurately weighed sample were extracted with acetone in a Soxhlet apparatus on a boiling water bath for 10 hr. The extracted material was dried under vacuum at 45°C to constant weight.

IV. RESULTS AND DISCUSSION

(*a*) *The Distribution of Leucoanthocyanins*

The analyses of leaves from young stems of different trees are given in Tables 3 and 4. The amount of leucoanthocyanin present decreased as the maturity of the leaves increased.

When a whole leaf was heated with the *iso*propanol–hydrochloric acid reagent, the reddest part was the tip. The pale tips were removed from each successive leaf

TABLE 3

DESCRIPTION AND LEUCOANTHOCYANIN CONTENT OF EUCALYPTUS SIEBERIANA LEAVES

Results calculated on volatile-free leaves

Leaf No.	Leaves Collected 15.xi.54			Leaves Collected 24.xi.54						Leaves Collected 7.xii.54			Leaves Collected 14.xii.54		
				25-Ft Tree			4-Ft Tree								
	Colour	Length (in.)	Leuco-antho-cyanin (%)	Colour	Length (in.)	Leuco-antho-cyanin (%)	Colour	Length (in.)	Leuco-antho-cyanin (%)	Colour	Length (in.)	Leuco-antho-cyanin (%)	Colour	Length (in.)	Leuco-antho-cyanin (%)
1	Yellow-red	0·75	0·63	—	—	—	—	—	—	Yellow-red	0·75	2·26	Red	0·75	0·84
2	Red	1·0	1·16	Red	0·75	1·31	—	—	—	Red	0·75	2·06	Red	0·75	
3	Max. red	1·25	1·08	—		—	—	—	—	Red	1·125	1·91	Max. red	1·25	0·78
4	Green along midrib	2·0	0·82	Max. red	1·25	1·22	Max. red	1·0	1·04	Max. red	1·25	1·88	Red	1·75	0·68
5	Greener	2·75	0·64	—	—	—	—	—	—	Red	1·5	1·58	Red	2·0	0·69
6	Greener	3·5	0·49	Green along midrib	2·5	1·18	—	—	—	Red	1·75	1·70	Red	2·5	0·57
7	Greener	3·5	0·52	—	—	—	Red	2·5	0·68	Red	2·0	1·31	Green	2·75	0·48
8	—	—	—	Greener	3·0	0·60	—	—	—	Green along midrib	2·875	1·28	—	—	—
9	—	—	—	—	—	—	—	—	—	Damaged	—	—	—	—	—
10	—	—	—	All green	3·0	0·60	—	—	—	Weak red	3·5	0·14	—	—	—
12	—	—	—	—	—	—	All green	1·75	0·77	—	—	—	—	—	—
14	—	—	—	—	—	—	Green	—	0·22	—	—	—	—	—	—
16	—	—	—	—	—	—	Green	—	0·15	—	—	—	—	—	—

on a freshly-collected stem and heated in each case with the same proportion of the reagent; the intensity of colour showed that the amount of leucoanthocyanin in the leaf tip steadily decreased as the leaf became more mature.

The outer layers removed from different points of a young stem of *E. sieberiana* were analysed. The anthocyanidins produced by both leucoanthocyanins and anthocyanins showed that these substances were most highly concentrated at a point 7–8 in. from the third red leaf and they decreased gradually on either side of this point.

The two samples of bark collected from a *E. sieberiana* tree showing vigorous leaf growth (November 15, 1954; Table 5) contained more leucoanthocyanin than those collected from different trees later in the season (Table 5).

The leucoanthocyanin present in the cambial zone (Table 5) decreased as the season progressed and, at the same time, the nature of the samples of this zone changed from a gel to an increasingly "stringy" condition. The latter physical condition indicates that more mature cells were present. This association is similar to the decrease in leucoanthocyanin in leaves of increasing maturity. The production of pink colour in the first samples (November 15, 1954) of cambial zone may have been due to enzymatic oxidation of the leucoanthocyanins to anthocyanins under the slightly acid (pH 5·4) and aerobic conditions that existed. The occurrence of pink coloured wood in some freshly-cut billets of *E. regnans* and other species may be due to such a mechanism. Both "pale" and "pink" wood contain the same amount of leucoanthocyanin, but under the influence of light and air there is a greater tendency for those with the lowest pH (as low as 3·0) to form the bright pink colour.

The above evidence suggests that there is association between active cell division and leucoanthocyanin content. Further support was found in the examination of kino which Jacobs (1938) showed was produced in the cambial region as a result of injury and the healing of the wound involved initially, the abundant proliferation of the cambium cells, with the production of parenchyma cells. The leucoanthocyanin in *E. sieberiana* kino was high—8·36 per cent. in the solid sample and 6·45 per cent. in the liquid.

The leucoanthocyanin content of *E. sieberiana* heartwood was double that of the sapwood of the same tree. According to one theory of heartwood formation, the zone of origin of the extractives is at the sapwood-heartwood boundary where there is intense cell activity in the rays and vertical parenchyma just before heartwood formation. The sapwood and heartwood in various logs of *E. marginata* were selected for intensive investigation because in this species not only is there a very great increase in the amount of extractives when heartwood is formed but two distinct heartwood zones are often apparent. Therefore to test whether leucoanthocyanins could be the precursor of the extractives in this species, transverse discs were examined by estimating the leucoanthocyanin in 30-μ tangential sections taken every centimetre or less, and the results are shown in Table 6 and Figure 2. There was an increase at the sapwood-heartwood boundary, a fairly constant amount across the outer heartwood zone, and then a decrease (compare with decrease of pinosylvin in pine heartwood (Erdtman, Frank, and Linstedt 1951)). The amount in included sapwood (Section 7, sample B (Table 6)) was much less than elsewhere. However, no localized increase in leucoanthocyanin content was found at the sapwood-heartwood boundary, which,

TABLE 4

DESCRIPTION AND LEUCOANTHOCYANIN CONTENT OF EUCALYPT LEAVES

Results calculated on volatile-free basis

Leaf No.	*E. obliqua*												*E. regnans*			*E. maculata*		
	Leaves Collected 24.xi.54			Leaves Collected 2.xii.54			Leaves Collected 7.xii.54			Leaves Collected 14.xii.54			Leaves Collected 1.xii.54			Leaves Collected 14.xii.54		
	Colour	Length (in.)	Leuco-anthocyanin (%)	Colour	Length (in.)	Leuco-anthocyanin (%)	Colour	Length (in.)	Leuco-anthocyanin (%)	Colour	Length (in.)	Leuco-anthocyanin (%)	Colour	Length (in.)	Leuco-anthocyanin (%)	Colour	Length (in.)	Leuco-anthocyanin (%)
1	Pink leaf	0·875	4·24	Yellow-brown	1·0	1·84	Yellow-brown	1·25	11·3	Pale green-red	1·25	0·67	Pink	0·375	3·49	Pink-green	1·0	0·078
2	Greener	1·5	2·82	Pink	1·5	1·90	Pink-red	1·0	>15	Greener	2·0	6·75	Red	0·625		Pink-green	1·0	
3	Greener	3·0	1·62	Pink-red	1·75	1·39	Pink	2·75	8·2	Green	3·75	4·5	Max. red	1·25	2·27	Red	2·0	0·096
4	—	—	—	Pink	2·5	1·63	Greener	3·25	7·0	—	—	—	Greener	1·625	2·24	Red	2·0	—
5	—	—	—	Greener	3·5	1·35	Green	—	8·0	—	—	—	Greener	3·0	1·02	Greener	3·0	0·012
6	—	—	—	Green	3·75	0·66	—	—	—	—	—	—	Greener	4·0	0·86	Greener	3·25	—
7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Greener	3·75	0·008
8	—	—	—	—	—	—	—	—	—	—	—	—	All green	3·0	0·76	—	—	—

if it had occurred, would have given a result similar to that obtained from the section including a kino vein (Section 11, sample A (Table 6)). If leucoanthocyanins are the precursors they are not formed in this region.

Table 7 gives typical analyses of different zones of *E. marginata.* The variation between the alkali soluble materials in the two heartwood zones are insignificant but the dissimilarity in the hot water soluble materials indicates a difference in constitution of the phenolic extractives. One explanation of the difference in these solubilities may be that the heartwood extractives are formed from low molecular weight substances and their conversion to the usual insoluble substances has in these abnormal cases been delayed. If this is true then the analyses of the 50-yr old jarrah samples indicate that the conversion has not commenced. In view of their water solubility the outer heartwood extractives could have been transported in the sap to the sapwood-heartwood boundary.

Table 5

LEUCOANTHOCYANIN CONTENT OF DIFFERENT TISSUES OF EUCALYPTUS SIEBERIANA AND E. OBLIQUA

The different values refer to samples from different parts of the tree

Date Collected	Leucoanthocyanin Content (%)				
	E. sieberiana				*E. obliqua*
	Bark	Cambium	Sapwood	Heartwood	Bark
15.xi.54	1·80, 1·72	1·24, 1·28, 1·36, 1·40	0·043, 0·044, 0·050	0·10, 0·10	—
24.xi.54					
25-ft tree	0·25, 0·37	—	—	—	0·057
4-ft tree	0·15, 0·20	0·32, 0·31	—	—	—
7.xii.54	0·53, 0·45	0·37 (base of trunk) 0·36 (top of trunk)	0·027	0·060	—

Alkali was necessary to remove all the heartwood extractives, but the hot water soluble and alkali soluble materials of the sapwood contained large amounts of non-phenolic substances not removed from the heartwood. If the alkali soluble materials present in the heartwood zones were considered to be polyphenolic substances, then a low percentage of leucoanthocyanins would be found in the materials occurring in the outer heartwood zone and a still lower one in the inner heartwood. On the other hand, the percentage of leucoanthocyanins in the acetone soluble material was high in the sapwood but decreased in the outer heartwood (Table 7).

Chromatographic examination showed that the anthocyanidins formed from the leucoanthocyanins did not decrease in any tissues to the same extent as the anthocyanins and flavone glycosides when the sample was taken at increasing distances from the leaves.

Both types of leucoanthocyanins were present in all differentiated tissues and they may well be formed initially in the leaves and transported along with carbohydrates and other materials to the rest of the tree. In support of this view, Lindner, Fitzpatrick, and Weeks (1950) found that any type of phloem blockage may produce polyphenol accumulation; from their description these polyphenols could have been leucoanthocyanins. Using staining and other techniques Wood (1933) found that the younger leaves of eucalypts and acacia contained the most tannin. At first it was distributed in all cells of the leaf but later was found only in the palisade and conducting tissues. The amount of tannin was found to decrease in the leaf as the quantity of lignin increased so that it was suggested that the phenolic nucleus in the tannin provided a starting point for the lignone complex of lignin. Wood also pointed out that considerable quantities of tannin in the leaf were transported to the bark. All of the polyphenolic substances mentioned in this present work would react similarly with the histological stains used by Wood.

Table 6

ANALYSIS OF SECTIONS TAKEN FROM TRANSVERSE DISCS OF EUCALYPTUS MARGINATA

Section	Leucoanthocyanin Content (%)		Section	Leucoanthocyanin Content (%)	
	Sample A	Sample B		Sample A	Sample B
1	1·50	1·51	9	3·35	3·00
2	1·58	1·30	10	2·82	1·65
3	3·43	3·01	11	4·35	2·50
4	4·13	2·83	12	2·24	1·60
5	4·62	2·42	13	4·10	2·12
6	4·56	2·79	14	1·44	1·86
7	3·19	0·20	15	2·34	1·15
8	4·00	2·10	16	4·61	—

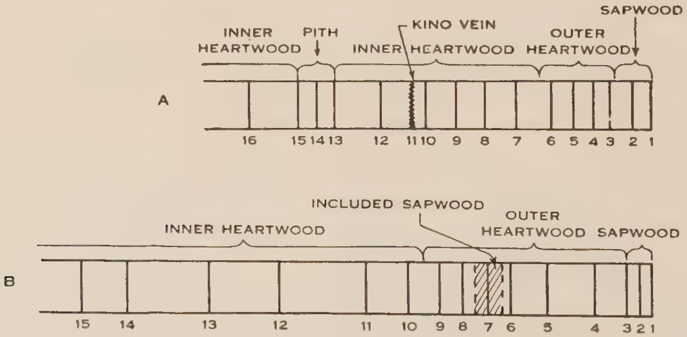


Fig. 2.—Locations of analytical sections in *E. marginata* log cross sections.

Leucoanthocyanins appear to be one of the products accumulated by unhealthy tissue as Stearns and Hartley (1952) found that virus-affected peach root gave a violet colour with methanolic hydrochloric acid. This colour is given by materials containing leucoanthocyanins in the presence of coniferyl aldehyde or wood lignin (Adler 1951). Lindner *et al.* (1950) found that whereas virus-infected woody plants gave good reactions with their polyphenolic stain, the virus-infected herbaceous plants gave no reaction; a similar observation has been made regarding the distribution of leucoanthocyanins in healthy tissues (Bate-Smith 1953).

TABLE 7

ANALYSIS OF EUCALYPTUS MARGINATA (JARRAH)

Results calculated on volatile-free wood. S = Sapwood, O = outer hardwood, I = inner hardwood

Sample	Zone	(1) Hot Water Solubles (%)	(2) 0·1N NaOH Solubles (%)	(3) Acetone Solubles (%)	(4) Leuco-anthocyanins (%)	$\frac{(4)}{(3)} \times \frac{100}{1}$ (%)
A	S	17·8	31·9	4·81	2·44	50·7
	O	18·4	29·0	12·2	2·45	20·1
	I	8·0	30·8	4·14	1·64	—
B	S	15·3	29·2	2·58	0·85	32·9
	O	15·9	30·9	16·1	2·90	18·0
	I	12·1	28·7	9·17	2·37	—
C	S	14·4	26·6	2·21	1·49	67·4
	O	9·1	26·9	10·5	0·93	8·80
	I	1·8	28·4	0·71	0·40	—
D	S	18·1	28·3	4·80	1·40	29·2
	O	15·4	30·8	11·8	3·30	28·0
	I	9·75	30·8	5·86	2·60	—
E	S	15·8	31·7	3·60	2·74	76·2
	O	8·9	31·0	19·4	2·30	11·8
	I	1·6	29·7	4·63	0·56	—
Regrowth 1	S	10·8	25·9	1·15	—	—
	I	31·2	39·7	—	—	—
Regrowth 2	S	14·6	28·0	2·25	0·35	15·6
	O	26·7	37·4	7·94	2·69	33·9
	I	26·2	36·3	5·36	1·95	—
Regrowth 3	S	14·0	24·8	0·83	0·54	65·1
	O	26·4	38·5	8·05	1·78	22·1
	I	25·5	34·7	4·69	1·78	—

(*b*) *Biogenesis of Flavonoid Materials and Tannins*

Different ratios of flavonoid materials were found after leucoanthocyanins were heated at 100°C under different conditions with *iso*propanolic and aqueous hydrochloric acid. For example, the conversion yield to anthocyanidin was reduced by the use of aqueous acid, or heating in an open tube, or heating for prolonged periods.

The leucoanthocyanins from *Acacia melanoxylon* readily change into other flavonoids, even when stored in an acetone solution in the dark (Hillis, unpublished data). When heated with *iso*propanol–hydrochloric acid for 40 min several substances were formed, amongst which 3, 3′, 4′, 7, 8-pentahydroxyflavylium chloride and 3, 3′, 4′, 7, 8-pentahydroxyflavone have been identified. A yellow fluorescent substance with $R_F = 0$ and leucoanthocyanin properties was also present. Swain (1954) also showed that the same classes of compounds are formed from leucocyanidin with dilute acid. Earlier, Forsyth (1953) found that, after 5 min heating at 100°C with 0·1N hydrochloric acid, leucocyanidin was changed to 31 per cent. (—) *epi*catechin, 6 per cent. (—) catechin, and other substances.

When 3, 3′, 4′, 7, 8-pentahydroxyflavylium chloride was heated with *iso*propanol–hydrochloric acid other flavonoids were produced. But with this substance heating for 3 hr was necessary before an 80 per cent. change occurred so that it does not affect the above observations. The only changes noticed with other classes of flavonoids were that quercetin yielded a dull yellow fluorescent compound ($R_F = 0$) on prolonged heating in an open tube with *iso*propanol–hydrochloric acid. Also kaempferol was formed when dihydrokaempferol was heated with 0·6N hydrochloric acid for a prolonged period.

Although the above *in vitro* conditions to which leucoanthocyanins have been subjected are severe, it is considered that enzymatic processes could accomplish the (and possibly other) transformations in the plant. Examples of leucoanthocyanins and other corresponding flavonoids occurring in nature are known. Chromatographic examination showed that the leucoanthocyanin and the corresponding 3, 3′, 4′, 7, 8-pentahydroxyflavone exist in *A. melanoxylon* heartwood. Forsyth (1952) isolated leucocyanidin, cyanidin glycosides, and catechins from fresh cacao bean extracts but the basic structure of these materials is ubiquitous in nature. Alston and Hagen (1955) have produced evidence that leucoanthocyanins were the precursors of the anthocyanins in the flowers of *Impatiens balsamina*.

The leucoanthocyanins are much more widespread in the plant kingdom than any other single class of flavonoid and this suggests that they may have some special function in the processes of the cell. They may play a part in the respiration of the cell as by appropriate dehydrogenation and dehydration of the C_3 portion of the molecule it is theoretically possible to transform leucoanthocyanin to most other classes of flavonoid materials. Apart from the *iso*flavones the two exceptions are the catechins and the benzalcoumaranones, the former could be formed by dehydration followed by hydrogenation, and the latter would have to be formed following a pyrone-ring opening in, say, a flavanol.

Szent-Györgyi (1937) and Reichel and Burkhart (1938) have considered that certain flavones play an important part as components of the oxidation-reduction systems of the plant. Hibbert (1941) proposed a scheme to indicate the course of the reductive and oxidative changes of C_6–C_3 units in the plant. Apparently the plant may selectively produce a given state of oxidation of the C_3 portion from some precursor which is characteristic of certain groups of plants. This implies that the hydroxylation pattern of the benzene rings is established prior to the oxidation (or reduction) of the C_3 portion to its final state and support for this is found in evidence collected by Geissman and Hinreiner (1952).

If the different classes of flavonoids are formed from leucoanthocyanins by an oxidation-reduction-dehydration process, then the same type of tissue in different plants would be expected to have similar sets of conditions favouring a certain class of compounds. However, this holds only for the class of flavanonols which are most often found in heartwood. Theoretically, flavanonols and flavonols can be formed sequentially from leucoanthocyanins, and they have been found together in nature with the following hydroxyl pattern: 3, 3′, 4′, 7-tetrahydroxy (Oyamada 1939); 3, 3′, 4′, 5, 5′, 7-hexahydroxy (Kotake and Kubota 1940); 3, 3′, 4′, 5, 7-pentahydroxy (Pew 1948); 3, 4′, 5, 7-tetrahydroxy (Hillis 1952); 3, 3′, 4′, 5′, 7-pentahydroxy (Freudenberg and Hartmann 1954).

The author has found that usually more than one anthocyanidin is formed when plant tissues are heated with the more efficient *iso*propanol–hydrochloric acid reagent (Hillis 1954 and unpublished work). The parent leucoanthocyanins could account for the occasional occurrence in the sample of representatives of the different classes of flavonoids with different hydroxyl pattern. The conversion to the different classes of flavonoids could depend on the nature of the leucoanthocyanin. For example, the phloem of *E. sieberiana* yielded delphinidin and cyanidin more slowly on treatment with *iso*propanol–hydrochloric acid than cambium gave cyanidin.

Eberhardt (1954) found that anthocyanin formation depended on intensity of respiration as shown by the rapidity of metabolism of sugar. The maximum amount of anthocyanin was formed after the maximum respiration occurred and he considered that colour formation took place during the last phase of a two-stage colouring synthesis, the second phase being independent of respiration metabolism. In this present work, the greatest amount of leucoanthocyanin was found in the youngest eucalypt leaves. Therefore, it is noteworthy that Arney (1947) found that there was a rapidly rising rate of respiration on young strawberry leaves as they unfolded and expanded. Similar observations have been made by Marsh and Goddard (1939) and James (1953).

(c) *Distribution of Materials Possibly Formed from Leucoanthocyanins*

By means of semi-quantitative comparisons in the chromatographic studies, changes in amounts of the resolved components have been observed.

Work in progress indicates that one of the main resolved components is quercetin glycoside. The amount contained in the petioles of the third red leaves was less than half the amount found in the leaves, and it was considerably less in the red external portion of the stem 2 in. away, and still less 12 in. away; the green portion of the stem at this latter position contained just detectable amounts of this substance which was absent from other tissues. Another component appears to be a myricetin glycoside and it was present in the leaves in about the same amount as the quercetin glycoside but it decreased more rapidly in the above tissues. On the other hand a mauve fluorescent substance with the properties of ellagic acid increased as the above two decreased.

The amount of anthocyanins in the red external portion of the stem 7–8 in. from the third leaf appeared to be greater than that in the leaf or elsewhere on the stem. The quantity of the two main flavone derivatives appeared to decrease more than the anthocyanins when the sample was taken in increasing distances down the stem.

The main red pigment in the external portion of the stem was different from those in the leaves. This observation has been confirmed with three different samples of *E. sieberiana*, and also on *E. obliqua* (3 samples), *E. maculata* (2 samples), *E. regnans*, *E. capitellata*, and *E. elaeophora*.

The author has shown (unpublished data) that the anthocyanins in the leaf are glycosides of delphinidin whereas the main one in the petiole and the external layer of the stem is a glycoside of cyanidin. It is possible that the delphinidin-based anthocyanins are retained by the leaf and the cyanidin-based anthocyanin is formed in the external layer of the stem. This view is supported by the observations that (i) frequently young stems had large areas of a red external layer although carrying few red leaves, and (ii) the injuries made by gall insects on pale pink leaves were surrounded by localized circles of intense red without any trace of movement into the veins.

The bark anthocyanins could be synthesized *in situ*, but it is also possible that a leucoanthocyanin originating in the leaf could be a precursor and be transformed to the anthocyanin under the influence of direct light and other agencies. The minor red colouring material in the outer layer of the stem appeared to be a delphinidin glycoside but there is no evidence that it originates in the leaves.

(*d*) *Origin of Heartwood Extractives*

The origin of the heartwood extractives is at present unknown. Some workers consider that they are formed from the starch in the sapwood cells during their sudden burst of activity prior to their death. Others consider that the extractives are transported centripetally from the cambium via the medullary rays (Jaccard 1938), but not transported centrifugally from the cambium into the bark as no trace of the heartwood phenols were found there (see Linstedt 1951). However, it has been shown in a number of eucalypts and other samples that the different transverse zones of the trunk and roots contained the same general pattern of resolvable materials (Hillis 1952).

In view of the evidence presented in this paper the author suggests that the leucoanthocyanins are the immediate precursors of the different classes of heartwood extractives based on the C_6–C_3–C_6 pattern and that these leucoanthocyanins originate in the leaves and principally the tips of the young leaves.

The leucoanthocyanins could give rise to different classes of compounds in response to different physiological conditions, diseases, temperature, or to light absorption. For example, conditions in the young leaves, the red external layer of the upper stem of certain eucalypts, and the callus tissues induced in the stems favour the formation of anthocyanins which are not found elsewhere in the tree. The transformation products are apparently left behind in these tissues which could be due to insolubility in the sap stream or to involvement in certain functions in that location. The leucoanthocyanins would need to join the stream of other materials in their passage from the leaves through the phloem to the sapwood. The saprophytic fungi considered to be associated with heartwood formation (Chattaway 1952) would be expected to establish a different oxidation-reduction potential to that existing in the phloem, and the leucoanthocyanins could be changed to different ratios of flavonoids and tannins in these two tissues.

This mechanism implies that the cambium does not play a part in the formation of heartwood extractives and there is support for this. Anderson (1953) found that the heartwood produced in pines as a result of stimulation of the cambium with sulphuric acid did not contain all the phenolic constituents of normal heartwood; also a different lignan has been found in wound resin (from the cambial region) and wound heartwood than has been found in the normal heartwood of certain spruce species (Erdtman 1949). Certain eucalypt kinos contain dihydrokaempferol (Hillis 1952, 1953) which has not been found in the heartwood of the same species. The argument against these cases is that the abnormal conditions induced may have been responsible for the differences. However, samples obtained during the normal growth of the tree show that leucoanthocyanins which yield cyanidin and delphinidin are present in all tissues other than the proliferating cambium and the kino formed in the cambium, whereas these latter contain only leucoanthocyanins which yield delphinidin. There does not appear to be any enrichment in the delphinidin: cyanidin ratio in the heartwood when compared with the phloem so the cambium does not supplement the supply of leucoanthocyanins (Table 2).

Numerous gaps in this thesis are being examined in order to test the validity of the postulations. The information should help in an understanding of the transformation of sapwood to heartwood, with the ultimate aim of control of heartwood formation, and, where required, an improvement in its durability.

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VI. References

Adler, E. (1951).—*Svensk PappTidn.* **54**: 445.
Alston, R. E., and Hagen, C. W. (1955).—*Nature* **175**: 990.
Anderson, A. B. (1953).—Int. Union Pure and Applied Chem. Stockholm. Abstr. Vol. p. 219.
Arney, S. E. (1947).—*New Phytol.* **46**: 68.
Barton, G. M., Evans, R. S., and Gardner, J. A. F. (1952).—*Nature* **170**: 249.
Bate-Smith, E. C. (1953).—*J. Exp. Bot.* **4**: 1.
Bate-Smith, E. C. (1954).—*Biochem. J.* **58**: 122.
Bate-Smith, E. C., and Lerner, N. H. (1954).—*Biochem. J.* **58**: 126.
Bauer, L., Birch, A. J., and Hillis, W. E. (1954).—*Chem. & Ind.* **1954**: 433.
Bottomley, W. (1954).—*Chem. & Ind.* **1954**: 516.

Chattaway, M. M. (1952).—*Aust. For.* **16**: 25.

Eberhardt, F. (1954).—*Planta* **43**: 253.

Erdtman, H. (1949).—*T.A.P.P.I.* **35**: 305.

Erdtman, H., Frank S., and Linstedt, G. (1951).—*Svensk PappTidn.* **54**: 275.

Forsyth, W. G. C. (1952).—*Biochem. J.* **51**: 511.

Forsyth, W. G. C. (1953).—*Nature* **172**: 726.

Freudenberg, K., and Hartmann, L. (1954).—*Liebigs Ann.* **587**: 207.

Geissmann, T. P., and Hinreiner, E. (1952).—*Bot. Rev.* **18**: 77.

Hibbert, H. (1941).—*Paper Tr. J.* **114**: 35.

Hillis, W. E. (1950).—*Nature* **166**: 195.

Hillis, W. E. (1952).—*Aust. J. Sci. Res. A* **5**: 379.

Hillis, W. E. (1953).—*J. Sci. Fd. Agric.* **4**: 135.

Hillis, W. E. (1954).—*J. Soc. Leath. Tr. Chem.* **38**: 91.

Isenberg, I. H., and Buchanan, M. A. (1945).—*J. For.* **43**: 888.

Jaccard, P. (1938).—Ber. Eidgen. Tech. Hochsch. Zürich No. 36.

Jacobs, M. (1938).—Bull. For. Bur. Aust., No. 20.

James, W. D. (1953).—"Plant Respiration." (Clarendon Press: Oxford.)

King, F. E., and Bottomley, W. (1954).—*J. Chem. Soc.* **1954**: 1399.

Kotake, M., and Kubota, T. (1940).—*Liebigs Ann.* **544**: 253.

Lawrence, W. J. C., Price, J. R., Robinson, G. M., and Robinson, R. (1939).—*Philos. Trans.* B **230**: 149.

Lindner, R. C., Fitzpatrick, H. C., and Weeks, T. E. (1950).—*Science* **112**: 119.

Linstedt, G. (1951).—*Acta Chem. Scand.* **5**: 129.

Marsh, P. B., and Goddard, D. R. (1939).—*Amer. J. Bot.* **26**: 724.

Oyamada, T. (1939).—*Liebigs Ann.* **538**: 44.

Pew, J. C. (1948).—*J. Amer. Chem. Soc.* **70**: 3031.

Pigman, W., Anderson, E., Fischer, R., Buchanan, M. A., and Browning, B. L. (1953).—*T.A.P.P.I.* **36**: 4.

Reichel, L., and Burkhart, W. (1938).—*Liebigs Ann.* **536**: 164.

Robinson, R. (1936).—*Nature* **137**: 172.

Robinson, G. M. (1937).—*J. Chem. Soc.* **1937**: 1157.

Robinson, G. M., and Robinson, R. (1933).—*Biochem. J.* **27**: 206.

Robinson, G. M., and Robinson, R. (1935).—*J. Chem. Soc.* **1935**: 744.

Robinson, R., and Robinson, G. M. (1939).—*J. Amer. Chem. Soc.* **61**: 1605.

Rutzler, J. E. (1939).—*J. Amer. Chem. Soc.* **161**: 1160.

Scott-Moncrieff, R. (1939).—*Ergebn. Enzymforsch.* **8**: 277.

Stearns, J. L. and Hartley, C. (1952).—*J. For. Prod. Res. Soc.* **2**: 58.

Swain, T. (1954).—*Chem. & Ind.* **1954**: 1144.

Szent-Györgyi, A. (1937).—*Biokhimiya* **2**: 151 (*Chem. Abstr.* **31**: 7894 (1937).)

Towers, G. N. H., and Gibbs, R. D. (1953).—*Nature* **172**: 25.

Wood, J. G. (1933).—*Aust. J. Exp. Biol. Med. Sci.* **11**: 139.

QUANTITATIVE ANALYSIS OF AMINO ACIDS USING PAPER CHROMATOGRAPHY

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Summary

A simple method for the quantitative analysis of amino acids present in a protein hydrolysate, or in other mixtures of amino acids, is described. The amino acids are separated on one-dimensional paper chromatograms, four solvent systems being used to resolve 17 amino acids. The chromatograms are treated with a suitable reagent to detect the spots corresponding to each amino acid. Each chromatogram, after being made semi-transparent with dimethylphthalate, is scanned automatically, with a densitometer, and the intensity of the light transmitted by the coloured spots is recorded on light-sensitive paper. A straight line relationship was found to hold, for all amino acids, between concentration and log per cent. transmission. The most useful range of amino acid concentration was 2–5 mM although the method is usable in the range 1–10 mM. The method includes a number of new techniques and the average coefficient of variation for a single reading for an amino acid is 5·7 per cent.

I. Introduction

Work in progress in this Laboratory on the composition of insect haemolymph required a method for the quantitative determination of amino acids in small volumes of biological fluids. Sufficient material is generally not available to enable the use of methods based upon microbiological techniques (Tristram 1953) or column chromatography (Moore and Stein 1949, 1951, 1954). For these reasons the development of a method for quantitative amino acid analysis by paper partition chromatography was undertaken.

Many papers have been published on the use of the techniques of paper chromatography for the quantitative analysis of amino acids in microgram quantities, and much of this work has been reviewed by Balston and Talbot (1952), Block, Durrum, and Zweig (1955), Kofrányi (1955), and Lederer and Lederer (1955). Additional references are given in the various sections below. The amino acids are separated from each other, identified, and the amount of each estimated. This estimation has been accomplished by:

(i) Measurement of the optical densities of the ninhydrin colours on the paper chromatograms (Brown, Kelly, and Watson 1953);

(ii) Elution of the amino acids from the paper followed by measurement of: (*a*) the optical densities of the ninhydrin colours (Chen and Hadorn 1954; Connell Dixon, and Hanes 1955; Gerok 1955); (*b*) nitrogen by the micro-Kjeldahl technique; (*c*) the optical densities of the soluble copper complexes;

(iii) Visual comparison of the ninhydrin colours on the papers with the colours given by known amounts of amino acids;

(iv) Measurement of the area of the ninhydrin spots;

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(v) A radiochemical method based on the incorporation of isotopes (Blackburn and Robson 1953).

Of these methods, development of and measurement of the intensities of the ninhydrin colours appears to be the most promising. It has been applied to both one- and two-dimensional, ascending and descending, chromatograms. In methods based on elution of the amino acids, or on measurements of the areas of the spots, difficulty is experienced in defining the boundary of an amino acid spot, more particularly if the spots lie close together.

The method we have developed is based on direct photometry on one-dimensional chromatograms of the coloured spots, formed by reaction of the amino acids with a suitable reagent. Scanning of the filter paper strips with a densitometer and recording of the intensity of the transmitted light on light-sensitive paper is done automatically. The majority of the published methods for the quantitative amino acid analysis by paper chromatography are unsatisfactory in so far as (1) the expected errors are too great or the method involves a large replication of analyses to obtain a reasonable degree of accuracy; (2) they are too complex for use as a routine analytical tool; or (3) insufficient details are given to enable the results to be repeated with the accuracy quoted.

II. Experimental Methods and Results

(a) *Reagents and Paper*

(i) *Amino Acids*.—All amino acids were checked for purity by paper partition chromatography. Particular attention was paid to samples of leucine which, it was found, may contain methionine.

(ii) *Phenol*.—B.D.H. "Analar" phenol was used without further purification. However, any samples which had acquired a pink colouration were rejected.

(iii) n-*Butanol*.—B.D.H. laboratory reagent *n*-butanol was used.

(iv) *Ninhydrin*.—A single large batch of B.D.H. laboratory reagent triketohydrindene hydrate was used throughout the entire work. It was checked for satisfactory colour yields for all amino acids.

(v) *Other Chemicals*.—Analytical reagent grades of acetone, isatin, and buffer reagents were used.

(vi) *Paper*.—Whatman No. 1 filter paper sheets were used for all experiments. In any one experiment or series of experiments it is considered advisable to use sheets from a single batch of paper. Except for buffering when required, the paper was not treated in any way. For quantitative work it is most important that the fingers do not come into contact with the paper surface being used in an experiment.

(b) *The Densitometer*

The densitometer was of simple construction. Light from a tungsten lamp (operated from a voltage stabilizer) passed through a filter and was "piped" through a "Perspex" rod and through a slit 8 × 2 mm to a Weston phototronic cell, model 594. The cell was connected directly to the terminals of the galvanometer in a photographically-recording Cambridge polarograph. The "Perspex" rod was covered loosely with black paper to prevent the entry of stray light. The paper strip to be scanned was drawn by a synchronous electric motor over the photocell, immediately beneath the "Perspex" rod. For ninhydrin colours a green filter was used which transmitted light in the region 560–590 mμ. For the histidine colour a green glass filter transmitting light in the region 500–550 mμ and for the isatin colours a red filter which transmitted light in the region 590–650 mμ were used. The light transmitted by the paper caused the photocell to produce a current which moved the coil of the galvanometer. The movements of the coil were recorded on photographic paper. There was a linear relationship between the intensity of light falling on the photocell and the current produced. The paper strip travelled at the rate of 2·54 cm per min and the photographic paper at the rate of 1·7 cm per min. This enabled the light transmitted by an entire strip of paper to be recorded on one piece of photographic paper. The light intensity was checked before and after each scan by measuring the amount of light transmitted by a suitable glass filter.

(c) *Chromatography*

All the amino acids appearing in a protein hydrolysate were separated in a series of one-dimensional chromatograms using the capillary-ascent method of Williams and Kirby (1948). For ease in handling, ease in replication, and for greater reproducibility the use of large sheets of filter paper was abandoned and the method was standardized on strips of filter paper 6·5 × 47·0 cm. Glass cylinders, 8 cm dia. × 55 cm high, were used as chromatogram chambers and these were kept in a constant temperature room at 25°C. Into each cylinder was placed 100 ml of the required solvent. To obtain good reproducibility every step in the procedure which follows must be rigidly standardized.

Three spots of the amino acid solution, instead of the usual one, were placed on the paper; for 5 μl spots, the centres were 1 cm apart on a straight line drawn 3 cm from one end of the paper strips, the central spot being in the middle of the paper. This technique produced, after development with solvent and colour development, "rectangular" spots *c.* 3 × 1 cm. When these coloured spots were scanned in the direction of their longer dimension it was shown that the intensity of colour was constant, or almost so, over a distance of *c.* 2·5 cm. Each paper strip was immersed to the same depth (*c.* 1 cm) in the solvent contained in the bottom of each cylinder. After the paper strips had been developed with solvent for the required time they were removed from the cylinders and dried. For phenol and butanol solutions this was accomplished by washing them in three separate lots of solvent ether and air drying. A batch of papers was washed a few at a time so that each paper strip was only immersed in ether for a short time. Clean ether was used for each experiment. Excessive washing in ether or the use of ether which had been used for washing papers for a previous experiment brought about variations in the amount of colour subsequently produced by the amino acids. When aqueous acetone was used as the solvent the papers were air dried.

The solutions used to detect the positions of the amino acid spots, i.e. for colour development, were allowed to flow on to the paper strips from a pipette. The pipette was not permitted to touch the paper. The paper strips were hung vertically, the bottom edge raised above the top edge, and the solution allowed to flow into the trough thus formed at the top of the paper. The bottom edge was slowly lowered so that the pool of liquid moved down the paper, more liquid being added from the pipette to maintain its volume. Surplus solution was removed from the bottom edge of the paper with the aid of a piece of filter paper. This procedure gave more reproducible results than did spraying the papers with the reagent solution and it was quite economical in regard to time and to the volume of solution needed (approx. 3·5 ml per 6·5 × 47.0 cm strip).

Three reagent solutions were used for the colour development:

(i) For proline and hydroxyproline, 1 per cent. isatin in ethanol was used, the treated papers being heated at 100°C for 10 min. Acetone is a less satisfactory solvent because of its greater volatility. Although the addition of acetic acid to the reagent considerably increased the intensity of the proline colour it gave less satisfactory results with hydroxyproline. It also gave colours with some other amino acids although these did not interfere. Heating the treated papers for a longer time at a lower temperature was not as satisfactory. The recommended method is not entirely specific for proline and hydroxyproline since glutamic acid also gives a dark colour (cf. Saifer and Oreskes 1954).

(ii) Histidine was detected with a diazotized sulphanilamide solution. Sulphanilamide (1 g) was dissolved in ethanol (*c.* 90 ml), concentrated hydrochloric acid (2 ml) was added, and the total volume made up to 100 ml with ethanol. Amyl nitrite (1·3 ml) was dissolved in sufficient ethanol to make 100 ml of solution. Both solutions were cooled to 0°C, equal volumes mixed, and allowed to stand at 0°C for 5 min. The diazotized solution was applied to the paper strips which were air dried and treated with 2N aqueous sodium carbonate. This formed a cerise colour with histidine. The papers were air dried. The above solution was found to be superior to those based on *p*-anisidine instead of sulphanilamide, or sodium nitrite instead of amyl nitrite. Aqueous sodium carbonate gave better colour development than did aqueous sodium or potassium hydroxide, aqueous ammonia, ethanolic sodium ethylate, ethanolic triethylamine, or buffers of pH 12.

(iii) Ninhydrin was used for the detection and estimation of all the other amino acids. The paper strips, after having been treated with an appropriate ninhydrin solution (*vide infra*), were allowed to air dry for a short period of time. Each paper strip was held at both ends in a frame so designed that the papers did not touch the frame except at each end, nor did they touch each other. The frame (with papers) was placed in an incubator at 20°C and 35 per cent. R.H. for 30 hr, by which time the colour development was complete (cf. Wellington 1952, 1953). Aqueous glycerol was used for humidity control.

After colour development was completed, the papers were prepared for automatic scanning in a densitometer. The strips were cut 5·5 cm wide (as required by the scanning apparatus), the coloured spots being centred in this width. Each strip was hung vertically and treated with redistilled dimethylphthalate using a pipette as described above. The paper strip was allowed to hang for a few minutes, blotted with filter paper to remove excess dimethylphthalate, allowed to hang for a further 2 or 3 min, and scanned immediately. Each paper strip was scanned twice, once on each side of the centre line. The two areas scanned were adjacent and were 8 mm wide. This was accomplished by moving the carrier of the densitometer, in which the paper travelled, over the photocell to pre-determined positions. The automatic scanning of each paper strip in the densitometer gave continuous graphs (see Fig. 1), each peak indicating the position of one amino acid and the height of each peak being a measure of the optical density of each spot. On each sheet of photographic paper is placed a line (AB) representing zero transmission (no light entering the photocell) the blank reading (CD) being obtained by recording the light transmitted at the beginning and end of each paper strip. Earlier work had shown that,

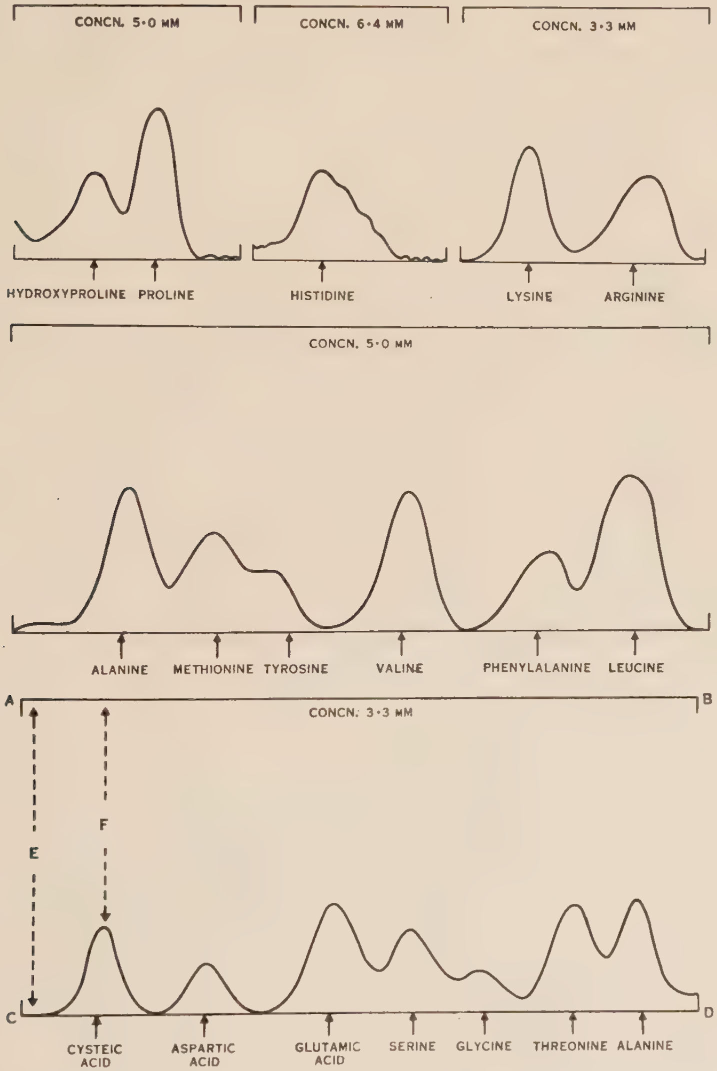

Fig. 1.—Optical transmission curves for mixtures of amino acids.

using the techniques and solutions described in this paper, blank strips of filter paper carried through the entire process gave, on scanning, a straight line parallel to the line representing zero transmission. Making the paper semi-transparent with dimethylphthalate has the effect of producing a straight line for such a blank instead of a wavy line. The percentage of light transmitted by the coloured spots is calculated by dividing the distance E by the distance F (see Fig. 1) and multiplying by 100. The value for E is the mean of the results from the two scans of each paper strip. In practice, both scans are recorded on the same sheet of photographic paper. A straight line relationship was found to hold for all amino acids between concentration and log per cent. transmission.

(*d*) *Solvents Used for Complete Separation*

Four solvents were used to give complete separation of the amino acids found in protein hydrolysates:

(i) Phenol (74 per cent. w/w) and a buffer of pH 10·0 (0·053M boric acid and potassium chloride, 0·047M sodium hydroxide) (cf. McFarren 1951). This solvent gave an excellent separation of cysteic acid, aspartic acid, glutamic acid, serine, glycine, threonine, and alanine. Large sheets of filter paper were hung up and saturated with buffer (pH 10·0) by allowing the buffer to flow on to the paper from a pipette. The sheets were air dried, cut into strips, and the amino acid solution applied. The strips were suspended in the cylinders containing the solvent and allowed to remain overnight so that they came into equilibrium with the atmosphere within the cylinders. The paper strips were lowered into the solvent and developed for 24 hr. For colour development, the strips were treated with 2 per cent. ninhydrin in ethanol containing 2 per cent. acetic acid. The papers were hung by the end where the amino acid solution had been applied before being treated with the ninhydrin solution. This minimized interference from a pale yellow stain which sometimes appeared near the solvent front. The stain was soluble in acidified ethanol and so tended to be washed from the paper.

(ii) *n*-Butanol (77 per cent. v/v), acetic acid (6 per cent. v/v), and water (17 per cent v/v). This solvent provided a good separation of alanine, methionine, tyrosine, valine, phenylalanine, and leucine. The amino acid mixture was first run overnight on unbuffered paper without prior equilibration. The papers were washed in ether, air dried, and run again overnight in the same solvent after the addition of 1·2 ml of a mixture of 10 per cent. aqueous ammonium molybdate (40 ml) and hydrogen peroxide (13·3 ml; 100 vol.) to each 100 ml of butanol-acetic acid-water. For colour development, the papers were treated with 1 per cent. ninhydrin in ethanol containing 0·25 per cent. triethylamine.

(iii) Lysine and arginine were separated on filter paper strips buffered at pH 7·0 (Sorensen phosphate buffer: 0·040M Na_2HPO_4, 0·027M KH_2PO_4) using 60 per cent. (v/v) acetone plus 40 per cent. (v/v) phosphate buffer pH 7·0. This solvent mixture had to be filtered before use as some salts were precipitated. The paper strips were developed overnight in the solvent, removed from the cylinders, and air dried. They were then developed in 80 per cent. aqueous phenol for 22 hr. For colour development, the papers were treated with 2 per cent. ninhydrin in ethanol.

(iv) Sixty per cent. (v/v) aqueous acetone gave a good separation of histidine, proline, and hydroxyproline. The paper strips were developed overnight in the solvent and air dried. Histidine and hydroxyproline were well separated and each paper strip was cut between these two amino acids. The histidine colour was developed on the lower part of the paper and the proline and hydroxyproline colours on the upper part of the papers.

(e) *Standardization of the Method*

Mixtures of all the abovementioned amino acids at concentrations of 0·005, 0·00417, 0·0033, 0·0025, and 0·002M were prepared in pH 10 buffer (boric acid-potassium chloride-sodium hydroxide) containing 10 per cent. *iso*propanol. The procedures for separation and estimation, described above, were carried out using eight to 10 replicates. An estimate of the reproducibility among replicates of optical density measurements for the amino acids is given in Table 1.

TABLE 1

REPRODUCIBILITY AMONG REPLICATES OF OPTICAL DENSITY MEASUREMENTS

Amino Acid	Coefficient of Variation for a Single Reading	Amino Acid	Coefficient of Variation for a Single Reading
Cysteic acid	3·2	Valine	4·5
Aspartic acid	5·7	Phenylalanine	7·1
Glutamic acid	5·2	Leucine	4·7
Serine	4·6	Lysine	8·9
Glycine	7·8	Arginine	6·3
Threonine	3·4	Histidine	9·9
Alanine	5·6	Proline	5·6
Methionine	5·5	Hydroxyproline	8·5
Tyrosine	1·4		

(f) *Amino Acid Analysis of a Simulated Insulin Hydrolysate*

The method of analysis was applied to a mixture of amino acids designed to reproduce approximately the composition of amino acids as found in a hydrolysate of the B chain of insulin (concentration corresponding to *c.* 1·25 g B chain per 100 ml; cf. Tristram 1953). To bring the concentration of the individual amino acids within the experimental range (viz. *c.* 2–5 mM) aliquots of the solution were diluted 1·5 and 3 times. For those amino acids separated by buffered phenol, the solution diluted 1·5 times was used except for glycine and glutamic acid. For these two amino acids and for those amino acids separated by *n*-butanol-acetic acid the solution diluted 3 times was used. The remaining four amino acids were estimated in the undiluted solution. Five replicates were used and the results are given in Table 2.

(*g*) *Recommended Procedure for Protein Hydrolysates*

After acid hydrolysis of a protein the excess acid must be removed by repeated evaporation *in vacuo*. Cystine, if present, must be converted to cysteic acid by the addition of hydrogen peroxide (100 vol., one-fifth of the volume of hydrolysate taken) to an aliquot of the hydrolysate, allowing the solution to stand for several hours, and evaporating to dryness *in vacuo*. All the hydrogen peroxide must be destroyed before phenol buffered at pH 10 is used as a developing solvent. If insufficient hydrolysate is available to enable an aliquot to be taken for the oxidation, the other three solvents are used first, the remaining hydrolysate treated with hydrogen peroxide and used with phenol as the developing solvent.

Table 2

COMPOSITION OF AN AMINO ACID MIXTURE

Amino Acid	Estimate from Regression Line (mM)	95% Confidence Limits	Approx. Standard Error	Actual Value
Cysteic acid	2·13*	2·33–1·90	0·09	2·25*
Aspartic acid	2·36*	2·65–2·02	0·14	2·21*
Threonine	2·56*	2·79–2·39	0·09	2·19*
Serine	2·60*	3·07–2·16	0·20	2·14*
Glycine	2·99†	3·26–2·74	0·11	3·32†
Glutamic acid	3·34†	3·69–3·03	0·14	3·32†
Alanine	2·40†	2·69–2·08	0·14	2·23†
Valine	3·36†	3·58–3·14	0·10	3·30†
Phenylalanine	3·54†	4·07–3·02	0·24	3·31†
Leucine	4·45†	4·76–4·16	0·14	4·32†
Tyrosine	2·22†	2·45–1·89	0·12	2·31†
Arginine	3·39	3·81–2·88	0·21	3·34
Lysine	3·61	4·08–3·15	0·21	3·34
Proline	3·33	3·58–3·08	0·11	3·35
Histidine	6·41	7·55–5·62	0·30	6·62

* Result to be multiplied by 1·5. † Result to be multiplied by 3·0.

III. Discussion

The success of quantitative analysis of amino acids by paper partition chromatography depends largely on the production of compact and discrete spots. The nature of the developed spots was found to depend on the original volume of solution applied to the paper, the number of such applications, the extent of equilibration, and on the technique used in applying the reagent. It is obvious that the smaller the original spot the more compact are the spots likely to be after development and best results were obtained when the original volume was small. The solubility of the amino acids in water absorbed by the paper leads to the formation of diffuse spots. This was demonstrated when spotted papers were hung for some hours in a moist atmosphere. If such papers were treated with ninhydrin, with or without development, the original spot was seen to have increased considerably in size, e.g. from 1·0 to 1·7 cm in diameter. Excessive drenching of the papers with a solution of ninhydrin in water-saturated n-butanol can also lead to tailing of the spots if the paper is suspended vertically during the operation. The procedure adopted, after testing several variations, was to apply ninhydrin (or other reagent) in absolute ethanol with the aid of a pipette, in order to ensure adequate and even distribution. Occasionally some spots, in particular aspartic and glutamic acids, appeared as ring-like structures or "halos". This appeared to be due to the super-imposition of aliquots of solutions to the original spot and single applications prevented a further occurrence of this source of error.

When buffered phenol at pH 12 was first used in this work, as recommended by McFarren (1951), the results were not satisfactory, as serine consistently had a low R_F value and it overlapped with glutamic acid. An explanation for this phenomenon is that the highly alkaline paper absorbed carbon dioxide causing a decrease in pH for, as McFarren has shown, separations at pH 11 are not satisfactory. Since from McFarren's tables a good separation was to be expected in the region pH 9–10, the paper and solvent were treated with a boric acid-sodium hydroxide buffer of pH 10·0. This procedure eliminated the trouble experienced at pH 12. The separation of glutamic acid and serine was further improved by bringing the paper strips into equilibrium with the atmosphere of the chromatographic chamber. If the paper is equilibrated in the same chamber as is used for the chromatographic run it removes from the solution considerable amounts of water. However, as variations in phenol content from 74 to 79 per cent. caused little, if any, alteration in the R_F values of the amino acids, the loss of water was not important in a single run provided the phenol content did not exceed 79 per cent. and the water could be replaced after each run if necessary.

In the analysis of those acids separated in n-butanol-acetic acid-water, use is made of the technique of developing the papers more than once in the same solvent. This procedure leads to better separations of the substances being chromatographed if the R_F values of those substances are less than 0·5. If the R_F values are greater than 0·5, the substances separated in the first development are brought closer together in each subsequent development (Csobán 1950; Jeanes, Wise, and Dimler 1951). The addition of hydrogen peroxide and ammonium molybdate for the second development in n-butanol-acetic acid-water converts the methionine to methionine sulphone. Methionine travels on the paper with valine, but methionine sulphone has a low R_F value (*c.* 0·08) and it finally appears between alanine and tyrosine. Residual traces of acetic acid on the paper strips cause the ninhydrin colours to be reddish. The addition of triethylamine to the ninhydrin solution produces blue spots with amino acids as also does the addition of other organic bases such as pyridine. Triethylamine was found to be the best base to use and the optimum concentration was 0·25 per cent. which gave a 20–25 per cent. increase in colour intensity when compared with the same ninhydrin solution without an organic base.

The method for estimation of the colour intensities involved the automatic scanning of the paper strips. When the paper strips were cut to fit the carrier of the scanning apparatus, it was not possible always to correct for non-vertical travel of the amino acids on the strips which sometimes occurred. Although this source of error was to some extent overcome by rigid standardization of the technique it was largely eliminated by the use of three spots of amino acid solution instead of the usual one. Slight variations in the direction of travel of this wide spot were not sufficient to cause any trouble. Statistical analysis of results showed that the three-spot method gave much greater precision than was obtained by use of single spots.

The method for the quantitative estimation of amino acids was standardized on replicate runs of five known concentrations which were approximately 5·00, 4·17, 3·33, 2·50, and 2·00 mM. For each of the amino acids the plot of the mean readings of the optical density readings of the spots against concentration appeared to be linear. This linearity was examined statistically and it was shown clearly that over the range of concentrations used there was no evidence of curvature for any amino acid.

An estimate of the reproducibility among replicates of the optical density measurements, each replicate being on a separate strip of filter paper, are given in Table 1. The average coefficients of variation for a single reading for each amino acid are recorded. In our hands the estimation of glycine has always been somewhat unsatisfactory. Statistical analysis of the results showed that the highest precision was obtained by measuring the maximum colour density of the spots and not by estimating the total colour of the spots (i.e. by measurement of the distance E and not by measurement of the area under the curve (Fig. 1)).

These calibration curves were used to estimate the concentrations of the amino acids in a mixture of amino acids designed to reproduce the composition of the hydrolysate obtained from the B chain of insulin. The results are given in Table 2 which also includes the 95 per cent. confidence limits (Eisenhart 1939) for the true concentration of each amino acid and the corresponding approximate standard errors. This procedure for testing the method was chosen because the composition of the "hydrolysate" was known accurately. As the composition of most biological materials varies with the source and as the method of hydrolysis introduces some unknown errors, the use of a biological preparation would not adequately test the method. The errors associated with the estimation of each amino acid are within the expected limits except for serine and threonine. These greater errors are caused by the high concentrations of glutamic acid and alanine (which travel adjacent to serine and threonine respectively) when compared with the concentrations of serine and threonine. This additional source of error will always occur when a small amount of one amino acid is not completely separated from a much larger amount of another amino acid travelling next to it on the paper strips. The error can be eliminated by adding a known amount of that amino acid present in low concentration to raise its concentration to approximately that of the interfering adjacent amino acid. The only error to be anticipated then is that indicated in Table 1. These results indicate very clearly that the coefficient of variation does not always give an indication of the accuracy of an analysis but only of its reproducibility.

Even though the same set of calibration curves can be used for analyses performed on different occasions, where a high degree of accuracy is desired calibration curves should be prepared at the same time as the unknown is analysed. If it is desired to correct for errors introduced by the method of hydrolysis, then the calibration curves would have to be prepared from hydrolysed amino acid mixtures;

however, the amino acid mixture would have to simulate the composition of the protein being analysed. Other amino acids than those mentioned can readily be estimated by slight modification of the described procedures. The isomeric leucines can be separated by use of a suitable solvent system, e.g. pyridine-amyl alcohol (Heynes and Walter 1951) and the coloured spots estimated on the papers. Tryptophan can be separated by a suitable solvent (e.g. *n*-butanol-acetic acid-water) and estimated by reaction with *p*-dimethylaminobenzaldehyde. The yellow colour formed with ninhydrin is not satisfactory. The papers are treated with a solution of *p*-dimethylaminobenzaldehyde (1 g) in a mixture of acetone (90 ml) and concentrated hydrochloric acid (10 ml) and allowed to air dry. Tryptophan gives a purple colour within a few minutes. Since the tryptophan colour changes on standing the colour intensity must be read after a fixed period of time. Asparagine and glutamine can be estimated in biological fluids by first estimating the concentrations of aspartic acid and glutamic acid, subjecting the amino acid mixture to hydrolysis (2N HCl at 100°C for 3 hr), and again analysing for aspartic acid and glutamic acid. The concentrations of asparagine and glutamine can be calculated from the difference in concentration of aspartic acid and glutamic acid before and after hydrolysis. By modifications such as these the concentration of any particular amino acid can be estimated. Although the most useful range of amino acid concentration is 2-5 mM the method is usable in the range 1–10 mM.

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V. REFERENCES

BALSTON, J. N., and TALBOT, B. E. (1952).—"A Guide to Filter Paper and Cellulose Powder Chromatography." (Reeve Angel and Co. Ltd., London, and Balston Ltd., Maidenhead.)

BLACKBURN, S., and ROBSON, A. (1953).—A radiochemical method for the microestimation of α-amino-acids separated on paper partition chromatograms. *Biochem. J.* **54**: 295–9.

BLOCK, R. J., DURRUM, E. L., and ZWEIG, G. (1955).—"A Manual of Paper Chromatography and Paper Electrophoresis." (Academic Press Inc.: New York.)

BROWN, G. L., KELLY, F. C., and WATSON, M. (1953).—"Nature and Structure of Collagen." (Ed. J. T. Randall.) pp. 117–21, 122–3. (Academic Press Inc.: New York.)

CHEN, P. S., and HADORN, E. (1954).—Vergleichende Untersuchungen über die freien Aminosäuren in der larvalen Haemolymphe von *Drosophila ephestia* and *corethra*. *Rev. Suisse Zool.* **61**: 437–51.

CONNELL, G. E., DIXON, G. H., and HANES, C. S. (1955).—Quantitative chromatographic methods for the study of enzymic transpeptidation reactions. *Canad. J. Biochem. & Physiol.* **33**: 416–27.

CSOBÁN, G. (1950).—Paper chromatography. *Mag. Kem. Fóly.* **56**: 449–51. (*Chem. Abstr.* **46**: 1384*f* (1952).)

EISENHART, C. (1939).—The interpretation of certain regression methods and their use in biological and industrial research. *Ann. Math. Stat.* **10**: 162–86.

GEROK, W. (1955).—Die quantitative papierchromatographische Bestimmung der Aminosäuren. *Hoppe-Seyl Z.* **299**: 112–28.

HEYNES, K., and WALTER, W. (1951).—Über Proteine und deren Abbauprodukte. V. Papierchromatographische Trennung der isomeren Leucine. *Hoppe-Seyl. Z.* **287**: 15–18.

Jeanes, A., Wise, C. S., and Dimler, R. J. (1951).—Improved techniques in paper chromatography of carbohydrates. *Anal. Chem.* **23**: 415–20.

Kofranyi, E. (1955).—Über quantitative Papierchromatographie von Aminosäuren in Proteinhydrolysaten. *Hoppe-Seyl. Z.* **299**: 129–38.

Lederer, E., and Lederer, M. (1955).—"Chromatography. A Review of Principles and Applications." (Elsevier Publ. Co.: Amsterdam.)

McFarren, E. F. (1951).—Buffered filter paper chromatography of the amino acids. *Anal. Chem.* **23**: 168–74.

Moore, S., and Stein, W. H. (1949).—Chromatography of amino acids on starch columns. Solvent mixtures for the fractionation of protein hydrolysates. *J. Biol. Chem.* **178**: 53–77.

Moore, S., and Stein, W. H. (1951).—Chromatography of amino acids on sulfonated polystyrene resins. *J. Biol. Chem.* **192**: 663–81.

Moore, S., and Stein, W. H. (1954).—Procedures for the chromatographic determination of amino acids on four per cent. cross-linked sulfonated resins. *J. Biol. Chem.* **211**: 893–906.

Saifer, A., and Oreskes, I. (1954).—Circular paper chromatography. II. Isatin as a colour reagent for amino acids. *Science* **119**: 124–5.

Tristram, G. R. (1953).—"The Proteins." Vol. 1A. Ch. 3. (Ed. H. Neurath and K. Bailey.) (Academic Press Inc.: New York.)

Wellington, E. F. (1952).—An ultramicro method for quantitative determination of amino acids. *Canad. J. Chem.* **30**: 581–91.

Wellington, E. F. (1953).—The effect of relative humidity on the reaction of ninhydrin with amino acids on paper chromatograms. *Canad. J. Chem.* **31**: 484–7.

Williams, R. J., and Kirby, H. (1948).—Paper chromatography using capillary ascent. *Science* **107**: 481–3.

STUDIES ON MARSUPIAL NUTRITION

I. RUMINANT-LIKE DIGESTION IN A HERBIVOROUS MARSUPIAL (SETONIX BRACHYURUS QUOY & GAIMARD)

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Summary

Some anatomical, digestive, and metabolic features of *Setonix brachyurus* Quoy & Gaimard, the Rottnest Island wallaby or "quokka", have been investigated. *Setonix* is a small macropod marsupial weighing 2–5 kg.

The complex stomach is large, sacculated, and has an oesophageal groove by-passing the sacculated region. Except in the oesophageal groove area, which has a stratified squamous epithelium, as in the ruminant, there is a columnar epithelium with tubular glands. The stomach contents amount to one-fifteenth of the body weight, and are six times greater than the contents of the caecum and upper colon.

The bacterial population of the stomach consists of some 15 morphological types, most forms being comparable with those in the rumen of sheep. Microbial attack on both cellulose and starch were observed.

Carbohydrates are fermented by these bacteria with the formation of volatile fatty acids (V.F.A.). The level of fatty acid varies according to feeding conditions from 2–14 m-moles per 100 ml "rumen" fluid. The pH varied in relation to these changes from 8·0–4·6. The gas of the stomach has a very high CO_2 content. Fermentation also occurs in the non-sacculated caecum and upper colon to a lesser extent.

Blood sugar determinations on five males indicate that the sugar level (15·8–78·9 mg per cent.) is more variable than in ruminants but of the same order. It is low compared with non-ruminants. V.F.A. was shown to be absorbed into the portal system, and some removed in the liver and by the tissues. At very low blood sugar levels (below 20 mg per cent.) the "body" appears to contribute sugar to the blood.

These findings, which are discussed and considered as an example of convergent evolution, show conclusively the ruminant-like digestion in the wallaby.

I. INTRODUCTION

A number of features converge to suggest that the digestion and the metabolism of carbohydrates in macropod marsupials is of a similar pattern to that found in ruminants (Moir *et al.* 1954).

There are a number of unpublished reports of cud chewing in kangaroos. However, Wood Jones (1923) reports that "no marsupial chews the cud, as do ruminants among the *Monodelphia*; but kangaroos and wallabies, as well as bandicoots, and probably some other forms have a curious habit of regurgitating their food. The animal, after a meal, makes a vigorous heaving movement of its chest and abdomen, and the stomach contents, which are forced up into the mouth, appear to be re-swallowed without any further chewing." Observations confirm this regurgita-

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tion which sometimes leads to ejection of a food bolus. We have also frequently observed chewing in kangaroos, usually in a lying posture, sometimes several hours after feeding. It therefore seems probable, but not proven, that they chew the cud or "ruminate".

The large size and sacculated condition of the wallaby stomach, together with the small flask-like caecum (Clark 1948), suggest that a pre-gastric bacterial fermentation of ingesta predominates, as in the ruminant, rather than the caecal fermentation of other herbivores. Figure 1 shows the general anatomy of the stomach region.

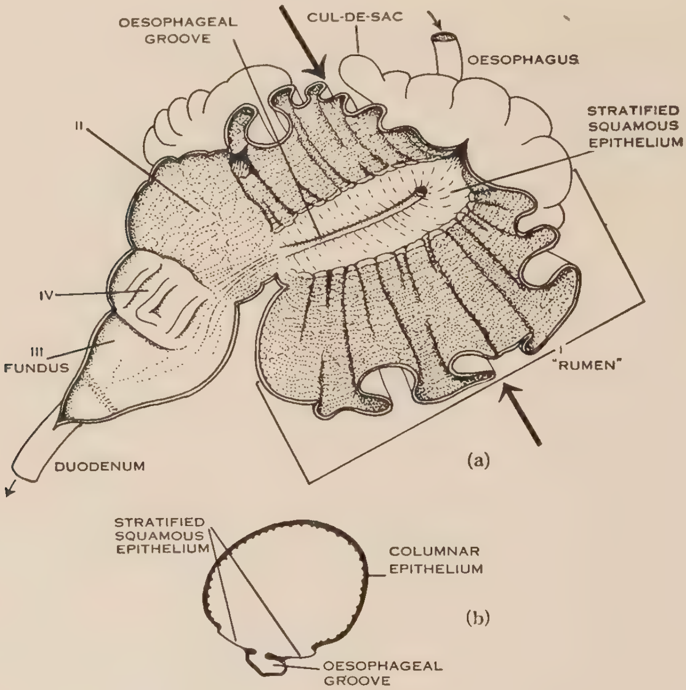

Fig. 1.—(*a*) General anatomy of the stomach of *Setonix*. (*b*) Transverse section of the stomach of *Setonix* at the arrows indicated in (*a*) showing the location of the oesophageal groove, stratified squamous epithelium, and columnar epithelium.

The fore-stomach, the putative rumen (region I), is sacculated and reflects above the oesophageal entrance to a cul-de-sac. A well-defined oesophageal groove runs from the oesophagus to the non-sacculated area (region II).

Unlike the rumen of sheep where the whole of the rumen, reticulum, and omasum has a stratified squamous epithelium, the epithelium of most of the stomach of the wallaby is columnar and possesses well-developed tubular glands. The only area of stratified squamous epithelium is the area about the oesophageal groove. Region III in Figure 1 is highly acidic and analagous to the abomasum of the ruminant. The functions of regions II and IV are not known. A more detailed account of the histology and anatomy of the alimentary tract will be published separately.

In ruminants, the fermentation of carbohydrates in the rumen restricts the glucose available for absorption. The low blood sugar level found in sheep, which can be lowered still further by insulin without overt ill-effect (Reid 1951), is already known to be paralleled in the wallaby (Buttle, Kirk, and Waring 1952).

A ruminant-type digestion with its unique exploitation of the synthetic and fermentative abilities of bacteria in the foregut, where the products of these activities are accessible to absorption, could explain the well-known ability of kangaroos and wallabies to survive on poor grazing.

Together, these considerations were considered sufficient to merit an exploratory investigation.

The possible parallel in the digestion and metabolism of carbohydrate between *Setonix* and ruminants was investigated on the following bases: (*a*) confirmation of bacterial fermentation in, and recovery of volatile fatty acids (V.F.A.) from, the stomach; and (*b*) recovery of V.F.A. from the blood with appropriate arterio-venous differences, coupled with low blood sugar levels.

II. Methods

The procedure adopted was to take the required blood samples, then examine gut contents for bacterial populations, fatty acids, pH, and weight of contents.

To obtain data from a variety of alimentary conditions, animals (*Setonix brachyurus* Quoy & Gaimard) were subjected to one of the following three sampling regimens:

Sampling was done at 10 a.m. and food was withdrawn either (1) 22 hr before sampling, or (2) 12 hr before sampling, or (3) about 6 p.m. and replaced at midnight. This latter treatment ensured a full stomach at sampling time, as the animals are night feeders. In all cases food consisted of commercial sheep nuts (mainly linseed and wheat products) and oaten chaff. Water was freely available at all times.

(*a*) *Alimentary Tract*

Samples for microscopical examination of gut contents were preserved by taking 10-g portions in 10 ml of 10 per cent. formalin. For this purpose samples from sites I and II (Fig. 1) were pooled, and samples from the caecum and the upper third of the colon were also pooled. The pH of fresh samples from these various sites was obtained by packing the contents about the electrodes of a Cambridge pH meter and allowing 2 min to equilibrate. V.F.A. were estimated in fluids expressed from fresh stomach or intestinal samples, which were distilled at constant volume in a Markham still as described by Elsden (1945) for rumen material. Results are expressed as m-moles acetic acid per 100 ml "rumen" fluid.

Ten-ml samples of gas were taken from two animals and passed through 10 per cent. KOH to determine the approximate CO_2 content. The residual volume was measured at constant pressure.

(*b*) *Blood*

Since the wallaby has no large superficial blood vessels, sampling involved dissection. At first concussion was used, and later anaesthesia with and without artificial insufflation. In the initial experiments using concussion, excitement hyperglycaemia was clearly involved. Blood drawn by rapid heart puncture was therefore used as a reference. Five ml of blood can be obtained in this way within 45 sec of first handling the animal. After intraperitoneal injection of "Veterinary Nembutal" (Abbott), the blood sugar values did not differ significantly from those of the heart puncture sample until 45 min after injection.

The procedure finally used was: injection of "Nembutal" (80–100 mg/kg) intraperitoneally into unexcited caged animals, rapid incision of the abdomen, injection of freshly dissolved heparin (10 mg) into the inferior vena cava (I.V.C.), dissection of the neck to expose the carotid artery, and, finally, withdrawal of 6 ml of blood from each site. Each sample as withdrawn was run into a bottle with oxalate (10 mg $K_2C_2O_4$). The limiting factor in speedy bleeding was the slow withdrawal from the veins and this necessitated the heparin treatment. All procedures were timed, and Table I shows a typical schedule.

Table 1

TYPICAL SCHEDULE* OF PROCEDURE FOLLOWED IN OBTAINING BLOOD SAMPLES

Animal weighed, anaesthetized with "Nembutal" (100 mg/kg)

Step No.	Procedure	Time (min)
1	Animal injected with "Nembutal"	0
2	Abdomen opened and heparin injected into inferior vena cava	8·45
3	Blood drawn from caudal inferior vena cava	11·15
4	Blood drawn from carotid artery	12·14
5	Blood drawn from hepatic vein	13·55
6	Blood drawn from portal vein	15·23
7	Chest opened and blood drawn from superior vena cava	16·45

* This schedule taken from records of July 24, 1953 for animal No. 23, weight 3·4 kg.

Before withdrawal of blood from the superior vena cava (S.V.C.) a clip was placed on the venous entrance to the heart. Although desirable, it was found impossible to clamp the hepatic vein (H.V.) near its entrance to the I.V.C. without delay and trauma, as this vein is very short. The H.V. samples may, therefore, have been diluted with some blood from the I.V.C.

Blood V.F.A. was determined by the method of Scarisbrick (1952), but using plasma instead of whole blood, since it was found that the red cells caused excessive difficulty and variability. V.F.A. values are expressed as mg acetic acid per 100 ml plasma. The identity of the V.F.A. was not determined. Blood glucose was estimated by the method of Somogyi (1937) using well-mixed whole blood.

III. Experimental Results

(a) *Alimentary Canal*

(i) *Weights of Ingesta*.—The weights of the stomach contents of 13 animals ranged from 80 to 485 g moist material. The mean weight was 207·5 g, whilst the mean weight of the animals was 3·16 kg. As some of these animals were not in a fully-fed condition this value for the weight of stomach contents must be considered conservative. It nevertheless represents approximately one-fifteenth of the total body weight.

On the other hand, the pooled contents from the caecum and the upper third of the colon, where water absorption had not influenced the consistency of the ingesta, averaged only 35 g, or one-ninetieth of the total body weight, and the fundus (region III, Fig. 1) contained only 15–20 g.

Table 2

pH AND VOLATILE FATTY ACID (V.F.A.) LEVELS OF INGESTA IN THE ALIMENTARY TRACT

Figures in parentheses are number of determinations

Sampling Regimen		Fore-stomach (rumen)		Post-stomach (fundus)		Caecum plus Upper Third of Colon	
		pH	V.F.A. (m-moles/ 100 ml "rumen" fluid)	pH	V.F.A. (m-moles/ 100 ml "rumen" fluid)	pH	V.F.A. (m-moles/ 100 ml "rumen" fluid)
1 (fasted 22 hr +)	Mean	7·58 (6)	2·25 (6)	2·38 (6)	0·32 (2)	7·00 (2)	3·9 (2)
	Range	7·05–7·95	1·8–2·6	1·76–5·42	0·31–0·33	7·00	3·4–4·4
2 (fasted 12 hr +)	Mean	7·12 (9)	3·57 (9)	2·75 (8)	0·52 (5)	6·8 (5)	6·9 (4)
	Range	5·75–8·00	2·1–6·5	2·00–6·30	0·35–0·75	6·0–7·35	4·4–9·6
3 (fully fed)	Mean	5·67 (6)	10·51 (6)	2·40 (6)	1·06 (2)	6·6 (2)	5·5 (2)
	Range	4·60–6·85	5·5–14·7	1·86–3·06	0·79–1·33	6·48–6·70	5·3–5·6

(ii) *Microscopical Examination of Stomach Contents.*—Microscopical examination of "rumen" contents showed a dense bacterial population strikingly similar to that of the sheep's rumen under similar conditions. This population consisted mainly of Gram-negative rods and cocci, with a few spiral forms. Gram-positive rods were also present and these dominated the population where the pH was below 5·5.

There were fewer forms of bacteria observed than for the sheep. Approximately 15 types were discernable compared with over 30 for the sheep (Moir and Masson 1952). Ciliates were not detected in any sample examined, either from the stomach, caecum, or colon. It is possible that this may have been the result of the diet, which may also explain the absence of many of the other striking components of the sheep's ruminal flora.

Cellulose and starch were both attacked by some of these organisms. The extent of cellulose digestion was not determined.

(iii) *Metabolic Data.*—The data in Table 2 demonstrate unequivocally that fermentation with the production of steam-volatile fatty acids occurs in the "rumen".

The level of fatty acid was clearly related to the time after feeding, varying from 2·25 m-moles per 100 ml "rumen" fluid in fasted animals, to 10·5 m-moles per 100 ml "rumen" fluid in those recently fed. The pH conditions bear a very close relationship to the level of acid (Fig. 2). The very low pH observed in some cases was probably related to lactic acid production. The upper pH level of the fasted animal (pH 8·0) was somewhat higher than that generally found in the rumen of sheep. The pH of the saliva was of the order of 8·5.

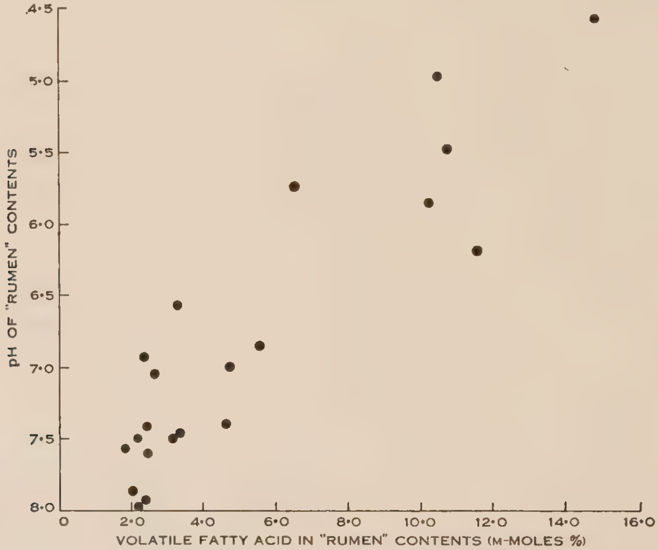

Fig. 2.—Relationship between volatile fatty acid levels and pH in the "rumen" of *Setonix*.

The very low level of V.F.A. in the fundus (0·32–1·06 m-moles per cent.) strongly suggests that the fermentation products were absorbed directly from the stomach as in the ruminant. Data from a single experiment support this, as the V.F.A. level was lower in region II (7·3 m-moles) than in region I (10·7 m-moles).

Large volumes of gas were not always found in the stomach, except during active fermentation, when the organ was usually distended. Gas samples taken from two fully-fed animals contained 60 and 75 per cent., respectively, of carbon dioxide. The nature of the residual gasses was not investigated.

Fermentation also occurs in the caecum and colon where both the pH and V.F.A. levels were found to be more constant (Table 2). The data indicate that a significant amount of V.F.A. was released in this region. Further, it appears that fermentation here was stimulated within a few hours of feeding, rose until at least 12 hr after feeding, then declined within 24 hr.

(*b*) *Blood*

A desirable summary of results would correlate V.F.A. titres in the alimentary canal with conditions in the bloodstream. This was not possible because, while our various methods of killing and drawing blood could not have introduced great differences into the conditions obtaining in the alimentary canal, hyperglycaemia intervened during the earlier experiments. For the blood samples, therefore, it seemed better to record only the figures for the final five animals for which all desirable precautions, indicated by previous experiments, were incorporated. The earlier results obtained are merely commented on where they indicate significant trends. Table 3 shows the V.F.A. and glucose titres collected under final conditions from five male animals.

TABLE 3

BLOOD PICTURE OF FIVE MALE SETONIX ANAESTHETIZED WITH "NEMBUTAL"

Sampling Regimen	Animal	Glucose or V.F.A. Levels (mg %)	Aorta	Inferior Vena Cava	Superior Vena Cava	Portal Vein	Hepatic Vein
1 (fasted 22 hr +)	A	Glucose	17·8	29·6	—	31·6	29·6
		V.F.A.	6·00	4·80	—	9·42	4·80
	B	Glucose	15·8	17·8	—	23·7	29·6
		V.F.A.	7·94	5·14	—	9·71	9·65
2 (fasted 12 hr +)	C	Glucose	25·6	21·7	33·5	43·4	45·36
		V.F.A.	5·94	5·94	2·17	4·68	8·97
3 (fully fed)	D	Glucose	45·4	29·6	45·4	84·8	76·9
		V.F.A.	11·36	4·51	5·25	34·89	22·04
	E	Glucose	78·9	39·4	65·1	63·1	96·6
		V.F.A.	7·02	2·34	1·83	17·8	14·51

The portal vein (P.V.) of animals on sampling regimen 3 carry a high V.F.A. and glucose content compared with the systemic circulation, but low glucose compared with non-ruminant herbivores. After passage through the liver (i.e. in the H.V.) the blood titre of V.F.A. has dropped. The glucose may have dropped or been elevated. One possible explanation for the consistent fall in V.F.A. concentration could be the conversion of some V.F.A. components to glucose. There is no direct evidence for this as the composition of the V.F.A. was not determined. Comparison of the concentrations in arterial and venous blood show that both glucose and V.F.A. were removed by the tissues. Whereas the "body" and the "head" reduced the concentration of V.F.A., equally, the "head" removed much less glucose.

Animals on regimen 1 showed a similar ratio of V.F.A. and glucose values in the various trunks except that comparison of I.V.C. and aorta levels on their face value imply that the body in these circumstances was contributing glucose to the blood.

The figures from the animal on regimen 2 were consistent with the liver contributing glucose to the blood. The "body", as in fasted animals, was taking very little V.F.A., and unlike those on regimen 1, was not contributing glucose to the blood. V.F.A. utilization appeared to vary directly with the level in the blood as indeed did the glucose utilization (Fig. 3).

Clearly, the measured blood sugar level was very dependent on feeding conditions in these experiments. Whether this applies to field conditions or other dietary regimens is not yet known.

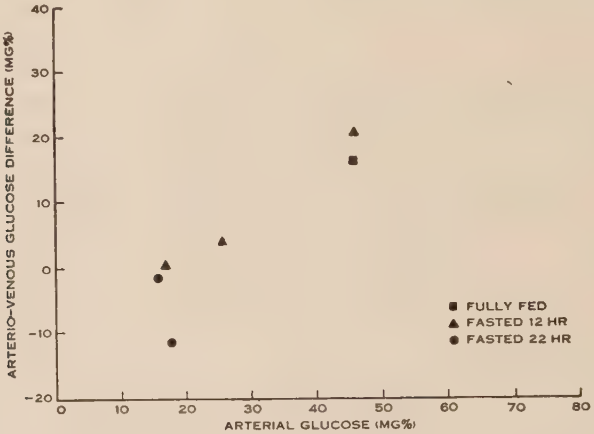

Fig. 3.—Relationship of blood glucose level to arterio-venous glucose difference in *Setonix*.

We have glucose values from another four fasted animals which were killed by concussion and blood drawn rapidly from the aorta, I.V.C., and P.V. These animals were part of an initial experiment performed in the belief that we could draw the blood samples before stress symptoms set in. In this we were wrong, but the fact that stress had supervened (as evidenced by raised blood sugar) makes the conditions worthy of note. The averages of recorded figures are: P.V.—49·5, aorta—66·7, and I.V.C.—53·6 mg per cent. Here, with the blood sugar augmented presumably from the liver (comparison of I.V.C. and P.V. showed that it was not from the gut), aortic blood sugar was higher than in the I.V.C. so there is no evidence of the body generally supplying sugar to the circulation.

In sampling regimen 2 there was information from five animals killed by concussion. There was no uniformity in sugar values so averaging of results would be meaningless. Where the aortic blood sugar was above 30 mg per cent., sugar was taken up by the tissues and the P.V. values obtained later showed a considerable increase in sugar due to stress. With the two animals where the aortic sugar was about 20 mg per cent. this same increase was not shown in the P.V., and the I.V.C. blood value was higher than the aortic, as was noted in the starved animals under "Nembutal" treatment. These figures, again, appear to imply that, where liver carbohydrate reserves are low, stress brings about release of muscle carbohydrate into the circulation.

IV. Discussion

In this exploratory investigation of digestion in *Setonix* many unexplained observations are recorded. However, a clear general picture emerges.

As in ruminants there is a capacious stomach region in which food is fermented by a dense bacterial population. The average ratio of stomach contents to large intestinal contents in *Setonix* is approximately 6:1. This is essentially the same as for ruminants, and the reverse of that in non-ruminant herbivores (Dukes 1947). The ratio of stomach content to body weight of 1:15 is, however, considerably less than for the larger true ruminants for which it is nearer 1:5 to 1:10 according to size (Elsden *et al.* 1946).

The stomach is not as clearly defined into four separate regions as in the ruminant, but has a well-defined oesophageal groove which by-passes the main fermentative region. Other points of difference are the columnar epithelium, and slightly higher fasting pH of the contents.

In ruminants, the rumen is buffered by bicarbonate, phosphate, and V.F.A. (Turner and Hodgetts 1955). A considerable quantity of both bicarbonate and phosphate is introduced into the rumen by the continuous and copious salivary flow (McDougall 1948). It is not known whether similar systems operate in the wallaby. *Setonix* does, however, exhibit copious salivary secretion under ether anaesthesia, and at high temperatures. It also has a strongly alkaline saliva.

Carbohydrates are fermented to V.F.A. by a bacterial population, apparently similar in composition and density to that of the sheep. The level of fatty acids is similar to that in ruminants (Elsden 1946). Their absorption is clearly indicated by the low level in the fundus (cf. Phillipson and McAnally 1942). The V.F.A. are absorbed into the P.V. directly from the upper stomach and probably also from the caecum (cf. Barcroft *et al.* 1944). The absence of information about the separate blood volume contribution of P.V. and hepatic artery to the total outflow from the liver in the H.V. makes it impossible to decide whether the disparity of V.F.A. values recorded is due to dilution or removal of V.F.A. by the liver. The difference in V.F.A. levels in these vessels is so great, however, that taken with the known events in the sheep, based on separate estimation of acetic and propionic acids, it seems likely that some fatty acid is being removed during vascular flow through the liver. Concurrently some glucose is absorbed from the alimentary canal, but both the intestinal and blood titres are low compared with those found in non-ruminant animals. There is clear evidence that both V.F.A. and glucose are utilized by the tissues, and, from the insulin experiments performed in another context (Buttle *et al.* 1952), it is known that only minimal quantities of blood glucose are essential for existence. The range of sugar levels (15·8–78·9 mg per cent.) is greater than that given for sheep by Reid (1950*a*), namely 18–57 mg per cent. A more marked hyperglycaemia after feeding occurred in these experiments. The V.F.A. levels and uptakes are essentially the same as in sheep (Reid 1950*b*) but the interplay between sugar and V.F.A. is not apparent from the limited data available. Exploratory dissection of other grazing macropods, e.g. *Bettongia*, *Protemnodon* ("tammar"), and *Macropus* (large kangaroos), shows similar alimentary anatomy. Stomach contents of a large kangaroo yielded a similar microbial population. It appears probable, therefore, that the general pattern of digestive physiology is similar in all macropods.

It is well known that in the non-ruminants usually studied, there is an alimentary hyperglycaemia after feeding, with a heavy load of sugar carried to the liver where glycogen is deposited. This glycogen serves as a carbohydrate reservoir from which, by interplay of hormones, notably insulin and adrenalin, the blood sugar is kept within the narrow limits required by the central nervous system. In the ruminant and in *Setonix*, which are able to survive on low blood sugar levels and can utilize V.F.A. as a metabolic fuel, a different pattern exists. The extent to which other mammals are able to use V.F.A., particularly in hypoglycaemia, is unknown. In some animals (e.g. man, dog, and pig) carbohydrate metabolism is dominant, and in others (e.g. sheep and wallaby) V.F.A. metabolism is dominant, whilst other herbivores (e.g. horse, rabbit, and guinea pig) are intermediate between these two types, or variable, according to the structure of the alimentary canal, or the level of fermentation in the large intestines. It is profitless to discuss this further until much more data from a range of animal species are available but it is appropriate to consider briefly (*a*) the merits of the ruminant-type digestion in colonizing geographic areas not capable of supporting mammals lacking pre-gastric digestive apparatus, and (*b*) the reasons for believing that the similar digestive physiology exhibited by sheep and *Setonix* is a clear-cut example of convergent evolution.

With regard to (*a*) three related features of ruminal-type digestion are of particular significance. These are cellulose utilization, nitrogen metabolism, and vitamin synthesis. Fermentation of cellulose in herbivores normally occurs to a limited extent in the caecum and colon. Digestion is not as efficient in these as in the rumen, largely because the rate of passage is too rapid to permit efficient bacterial action. In the horse, food is eliminated in 24–27 hr (Alexander 1946), whereas in the cow 80 per cent. is eliminated in 70–90 hr and the remainder in 7–10 days (Balch 1950). In the ruminant, removal of cellulose and growth of bacteria *precede* normal alimentary digestion and absorption. This enables the products of fermentative breakdown by bacteria, along with the products of bacterial synthesis and food nutrients enclosed within the plant cell walls, to become readily available for absorption.

These facts show clearly that pre-gastric bacterial digestion confers important benefits upon the animal. Energy from poor fibrous foods is more efficiently garnered and the available energy is yielded over a longer period of time so that the animal is more resistant to periods of starvation; the uptake of nitrogen and other nutrients is enhanced; and considerable synthesis of vitamins and essential amino acids can take place. Although the gross value of ruminant digestion is apparent, a study of its minutiae as a basis for understanding ecology has been largely neglected. Raven and Gregery (1946) have discussed the radiation of macropods in relation to habitat. This investigation, based on museum and field studies, shows the adaptive branching in relation to forest, thicket, and grassland. This now could profitably be supplemented by a comparative study of digestive physiology of various macropods and their ecologically equivalent eutherian ruminants. Already it is known that there is a broad similarity between the sheep and the wallaby.

At one time the mammals were considered monophyletic because it seemed unlikely that monotremes, marsupials, and Eutheria would have developed the mammalian features independently. This monophyletic origin is not supported by recent authors. With some variation in detail there is considerable agreement that there has been separate derivation from therapsid reptiles. According to Olson (1944) "it would appear that more than one line of therapsids, probably three to five, crossed the mammalian threshold and that the common ancestry of mammals lies well below the level of the first mammals, probably somewhere below the level of the upper Permian." To Olson the monotremes "appear to have little to do with other groups of mammals and must have had a history long independent." Simpson (1950) also favours a very early origin of monotremes and his view that they are really highly modified therapsid reptiles is epitomized in his description of them as "mammals by definition rather than ancestry." Watson (1953) expresses the opinion that monotremes could readily be derived, with small modifications, from tritylodont reptiles. Evidence from cytology (Matthey 1949) and pharmacology (Feakes *et al.* 1950) is consistent with monotremes having closer affinities with present day reptiles than with other mammals. Olson and Simpson agree in deriving marsupials and placentals from Pantotheria (Jurassic mammals) with a divergence between these two sometime during the Cretaceous.

Fossil evidence indicates that, of the "warm blooded" stocks that emerged from the therapsid reptiles, two survive to the present. These two—Prototheria (monotremes) and Theria (marsupials and Eutheria) have only a tenuous and remote relationship to each other but they possess many characters indicating convergent evolution. The marsupials and Eutheria, though having a distant common ancestral group (Pantotheria) have been separate since the Cretaceous; Eutheria are not derived phylogenetically from Marsupialia. It can be concluded therefore that adaptive modifications, e.g. the ruminant-type digestion found in these groups, have been independently and convergently arrived at and do not reflect phylogenetic relationships. Whilst not in any way invalidating this general conclusion, it would clearly be valuable to know, in the present context, whether marsupials, other than grazing macropods, have alimentary canals similar to those described here for *Setonix*. Dissection of koalas, Australian opossums, and bandicoots offers no support for this possibility, nor does Johnstone's (1898) description of a greater range.

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VI. References

Alexander, F. (1946).—*J. Comp. Path.* **56**: 266.

Balch, C. C. (1950).—*Brit. J. Nutrit.* **4**: 361.

Barcroft, J., McAnally, R. A., and Phillipson, A. T. (1944).—*J. Exp. Biol.* **20**: 120.

Buttle, J. M., Kirk, R. L., and Waring, H. (1952).—*J. Endocrinol.* **8**: 281.

Clark, J. R. (1948).—*J. R. Soc. W. Aust.* **33**: 59.

DUKES, H. H. (1947).—"The Physiology of Domestic Animals." 6th Ed. (Comstock Publ. Co.: New York.)

ELSDEN, S. R. (1946).—*J. Exp. Biol.* **22**: 51.

ELSDEN, S. R., HITCHCOCK, M. W. S., MARSHALL, R. A., and PHILLIPSON, A. T. (1946).—*J. Exp. Biol.* **22**: 191.

FEAKES, M. J., HODGKIN, E. P., STRAHAN, R., and WARING, H. (1950).—*J. Exp. Biol.* **27**: 50.

JOHNSTONE, J. (1898).—*J. Linn. Soc.* (*Zool.*) **27**: 1.

MCDOUGALL, E. I. (1948).—*Biochem. J.* **43**: 99.

MATTHEY, R. (1949).—"Les Chromosomes des Vertebres." (F. Rouge: Lausanne.)

MOIR, R. J., and MASSON, M. J. (1952).—*J. Path. Bact.* **64**: 343.

MOIR, R. J., SOMERS, M., SHARMAN, G., and WARING, H. (1954).—*Nature* **173**: 269.

OLSON, E. C. (1944).—*Spec. Pap. Geol. Soc. Amer.* **55**: 1.

PHILLIPSON, A. T., and MCANALLY, R. A. (1942).—*J. Exp. Biol.* **19**: 199.

RAVEN, H. C., and GREGERY, W. K. (1946).—*Amer. Mus. Novit.* **1309**: 1.

REID, R. L. (1950*a*).—*Aust. J. Agric. Res.* **1**: 182.

REID, R. L. (1950*b*).—*Aust. J. Agric. Res.* **1**: 338.

REID, R. L. (1951).—*Aust. J. Agric. Res.* **2**: 132.

SCARISBRICK, R. (1952).—*Biochem. J.* **50**: xxxiv.

SIMPSON, G. G. (1950).—"Meaning of Evolution." (Yale Univ. Press: New Haven.)

SOMOGYI, M. (1937).—*J. Biol. Chem.* **117**: 771.

TURNER, A. W., and HODGETTS, V. ELIZABETH (1955).—*Aust. J. Agric. Res.* **6**: 125.

WATSON, D. M. S. (1953).—*Evolution* **7**: 159.

WOOD JONES, F. (1923).—"The Mammals of Southern Australia." Pt. I. p. 59. (Govt. Printer: Adelaide.)

STUDIES IN THE METABOLISM OF PLANT CELLS

XI. THE DONNAN EQUILIBRATION AND THE IONIC RELATIONS OF PLANT MITOCHONDRIA

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Summary

Ionic relations of mitochondria from red beetroot tissue (*Beta vulgaris* L.) suspended in various salt media were studied. Under the conditions of the experiments the mitochondrial suspensions behaved as a two-phase system, one containing immobile ions and showing Donnan effects. Some differences between mitochondria which showed simple Donnan characteristics and those accumulating chloride ions are described.

Salts extracted from the mitochondria by 5 per cent. formaldehyde followed more closely the Donnan expectation than the total salt content or salts extracted by 5 per cent. trichloroacetic acid following the extraction with formaldehyde. External potassium readily replaced mitochondrial sodium but external sodium had little effect on potassium content of the mitochondria. The beetroot mitochondria are another instance of biological systems distinguishing between these two superficially similar elements.

The potassium, sodium, calcium, phosphate, chloride, nitrogen, water, and dry weight contents were determined on mitochondria isolated from beetroot tissue in different ways and suspended in different media. A comparison with the constitution of cat-heart sarcosomes is given. The effect of pH during extraction of mitochondria from the beetroot tissues was investigated. The possibility of active transport of cations as well as anions is suggested.

I. Introduction

The cell is a complex multiphase system. Since many of the cellular contents, particularly the plastids and mitochondria, are characterized by membranous structures (Mercer *et al.* 1955; Farrant *et al.* 1956), partitioning of the internal salt content will occur between them and the cytoplasm. Further, the possibility that mitochondria mediate active salt accumulation in cells has been suggested (Robertson 1951) and evidence for active accumulation of chloride ions by mitochondria has been obtained (Robertson *et al.* 1955).

As was pointed out by Robertson (1956) interpretations concerning the salt absorption by plants have been confused by not separating the Donnan adjustment and active accumulation processes of salt absorption. Such a confusion may also occur with salt uptake by mitochondria. Steinbach (1951) has pointed out that salt entry must be shown not to occur by simple diffusion before more complex explanations are invoked. Concentrations of potassium and sodium in mitochondria higher than in the external medium have been found (Macfarlane and Spencer 1953; Bartley and Davies 1954; Robertson *et al.* 1955). Although the internal concentrations were

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influenced by changes in metabolism, in no case was physical equilibration shown inadequate to explain the concentration differences. Indeed, data concerning both plant and animal mitochondria (Macfarlane and Spencer 1953; Spector 1953; Robertson *et al.* 1955) suggest that a Donnan equilibration of potassium and sodium between external media and the mitochondria predominates. This is further supported by the external-internal concentration relation of chloride ions of plant mitochondria (Robertson *et al.* 1955) where a Donnan adjustment at high external chloride concentration can adequately explain the masking of the active accumulation of chloride.

The results in this paper concern a more detailed study of the ion balance in beetroot mitochondria where active accumulation of chloride was usually low.

II. Methods and Materials

(*a*) *Materials*

Heterogenous tissues of red beetroot (*Beta vulgaris* L.) from a commercial source were used to supply plant mitochondria.

(*b*) *Isolation of Mitochondria*

Modifications of the methods previously described (Robertson *et al.* 1955) were used. All steps were carried out in the cold.

The red beetroot was chilled in a cold-room (−5–5°C) and diced into *c.* 0·5 cm cubes. The diced beetroot was disintegrated in chilled (<5°C) 0·4M sucrose in a Waring Blendor for 30 sec at the rate of 100 g/110 ml sucrose. Usually 400 g of beetroot tissues were disintegrated in each experiment. In some experiments ethylenediaminetetra-acetic acid (EDTA) and tris(hydroxymethyl)aminomethane (TRIS) were also added to the extraction media.

The brei was filtered through muslin cloth and the filtrate centrifuged for 5 min at 900 *g*. The residue containing cell debris was discarded. The supernatant was recentrifuged for 25 min at 5000 *g* in a refrigerated centrifuge at −2°C. The supernatant, generally pH 5·8, from the higher speed centrifugation was discarded. The residue consisted mainly of mitochondria but possibly contained microsomes and fragmented starch grains and nuclei. Examination of the residue by electron microscopy, either with shadowed, dried suspensions or with sections of osmium tetroxide-fixed mitochondria, suggests a low contamination. For purposes of convenience the mixture will be called "mitochondria".

The mitochondria from high-speed centrifugation were resuspended in the cold-room in chilled 0·4M sucrose containing different amounts of salts. Usually, as six centrifuge tubes could be manipulated simultaneously, the mitochondria from 400 g beetroot were divided into six portions for different treatments in each experiment. Each portion contained about 100–200 mg, fresh weight, of mitochondria. Details of variations from the above procedures will be given as required.

(*c*) *Respiration of Mitochondria*

The oxygen uptake of the mitochondria resuspended in the various salt media without buffer or exogenous substrate was measured as a routine by Warburg's direct method at 25°C. The results are expressed as Q_{O_2}(N) (uptake of oxygen in μl/hr/mg mitochondrial nitrogen) and represent the final respiration rates after about 60 min, not initial rates. The final rates probably represent more closely the respiration of the mitochondria adjusted to conditions of incubation in salt media at the time of their analyses.

(*d*) *Ion Balance*

The ion content of the mitochondria suspended in various media was determined by analyses of extracts of the mitochondria rapidly sedimented at high relative centrifugal forces for short times. All values are corrected for 50 per cent. v/v extra-mitochondrial liquid in the mitochondrial pellet after decantation of supernatant and removal of excess liquid from the centrifuge tubes (Robertson *et al.* 1955). For example, the corrected value on a volume basis, $c_{i_{50}}$, equals $2c_i - c_0$, where c_0 is the external concentration and c_i is the total salt content of the mitochondria plus extra-mitochondrial liquid per pellet volume. The pellet volume was calculated from the pellet weight, assuming the density to be at least the density of the suspending medium. Although the mitochondrial content of the pellet varies around 50 per cent. by volume (Robertson *et al.* 1955), this correction brings the data into closer agreement with the actual internal mitochondrial salt content. Calculations of the salt content per mitochondrial nitrogen are also given.

After holding at 25°C in the Warburg vessels, generally for 1 hr, 4-ml aliquots of the suspensions were placed in six high-speed centrifuge tubes and centrifuged at room temperature for 5 min at 36,000–48,000 *g*. The supernatants were decanted and retained for analyses for external salts. Excess liquid was removed with filter paper from the high-speed centrifuge tubes containing the mitochondrial pellet. The tubes were then weighed to obtain the mitochondrial pellet weights.

To each tube containing the mitochondria 5 ml of extracting solutions were added. The tubes plus mitochondria plus extracting solutions were weighed. After the mitochondrial pellets were resuspended, the suspensions were centrifuged for 5 min at 38,000–72,000 *g*. The extract supernatants were decanted and retained for analyses. Excess liquid was removed with filter paper from the high-speed centrifuge tubes, and the tubes containing the extracted mitochondria weighed. For some experiments the mitochondria were extracted a second time following the procedure above, the extracted residues dried over $CaCl_2$, and their weights determined.

Extracting solutions were trichloroacetic acid (TCA) and formaldehyde (HCHO), both 5 per cent. The "HCHO" fraction contained salts obtained with HCHO, and the "TCA" fraction contained salts extracted with TCA after prior extraction with HCHO. References to "total" salts are concerned with salts obtained with TCA as the sole extracting solution or the sum of the HCHO and TCA extractions. TCA was chosen because it was thought it would disintegrate the particles and remove most of the bound ions. HCHO was used because it was thought it would cause less disintegration of the particles and allow only the mobile constituents to diffuse out. The validity of these assumptions will be referred to later.

Analyses for both potassium and sodium, and for chloride, were carried out by flame photometry and electrometric titration respectively (cf. Robertson *et al.* 1955).

For assays in which calcium was also determined, flame photometry was employed with standard reference solutions approximating the content of the unknowns (Na+K+Ca) without adding sodium or potassium to the unknowns. Mitochondrial nitrogen and phosphate were determined by difference between the mitochondrial suspensions and the supernatants from high-speed centrifugation (38,000–72,000 *g*). Aliquots of mitochondrial suspensions and the supernatants were digested in H_2SO_4 and decolorized with 30 per cent. H_2O_2. The diluted digests were used to assay for nitrogen by Nesslerization and total phosphate by the method of Lowry and Lopez (1946).

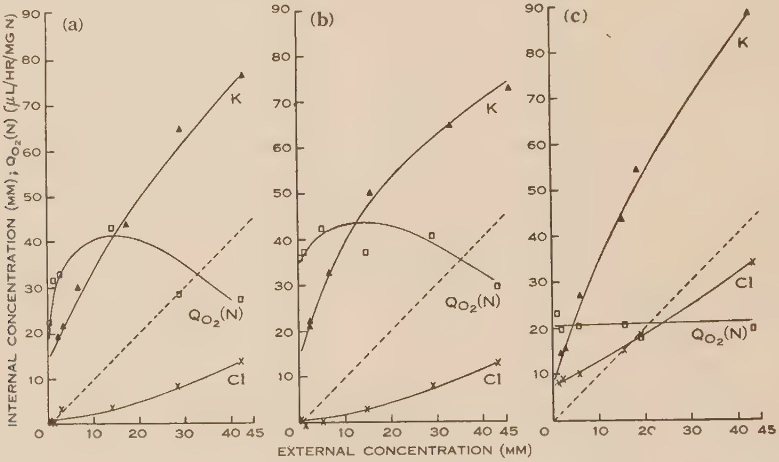

Fig. 1.—(*a*) and (*b*) The Donnan equilibration of mitochondria incubated in various external concentrations of potassium and chloride, and (*c*) chloride accumulation imposed upon the Donnan equilibration. Respiration of the mitochondria as functions of external chloride concentrations. ---- Equality of internal with external concentration. Each set of points for (*a*), (*b*), and (*c*) represents a different mitochondrial preparation.

III. Results

(*a*) *Accumulation of Chloride*

Earlier work showed that mitochondria from beet tissue can accumulate chloride from dilute solutions. Further work (Honda and Robertson, unpublished data) has shown that the amount of this accumulation varies with the time of the year that the mitochondria are extracted from the beet. No attempt will be made to report these seasonal results here, but the differences are illustrated by comparison of the chloride data in Figures 1(*a*) and 1(*b*) with those in Figure 1(*c*). In some experiments the internal chloride concentration exceeded the external but in others it was less as would be expected from the Donnan equilibrium.

(*b*) *The Donnan Equilibration*

Figures 1(*a*) and 1(*b*) show the total internal potassium and chloride concentrations of mitochondria as functions of external concentrations. The mitochondrial system superficially resembled the partitioning of KCl expected from the following equations describing a two-phase Donnan system containing only mobile uni-univalent salts:

$$[\mathrm{K}^+]_i = \tfrac{1}{2}[(A^2+4c_0^2)^{\frac{1}{2}}+A],$$

$$[\mathrm{Cl}^-]_i = \tfrac{1}{2}[(A^2+4c_0^2)^{\frac{1}{2}}-A],$$

where $[\mathrm{K}^+]_i$ and $[\mathrm{Cl}^-]_i$ are the concentrations of the mobile positive and negative ions, respectively, in phase i, A the concentration of immobile anions in phase i, and c_0 the concentration of the negative and positive ions in phase 0, which is comparatively very large.

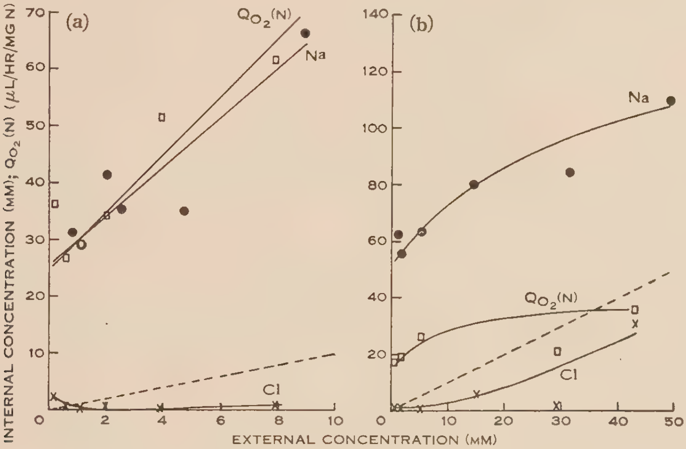

Fig. 2.—(*a*) and (*b*) The Donnan equilibration of mitochondria incubated in various external concentrations of sodium and chloride. Respiration of the mitochondria as functions of external chloride concentration. ---- Equality of internal with external concentration. Each set of points for (*a*) and (*b*) represents a different mitochondrial preparation.

The mitochondrial suspensions containing added sodium chloride instead of potassium chloride also showed the resemblance to the Donnan equilibration (Figs. 2(*a*) and 2(*b*)). The respiration rates were variable with time and thereby differed from those of mitochondria which accumulated chloride more actively (Fig. 1(*c*)).

(*c*) *Potassium-Sodium Replacement*

Some decrease in total internal sodium concentration accompanied the increase of the external and internal potassium concentrations (Figs. 3(*a*), 3(*b*), 3(*c*)); i.e. as expected from the Donnan equilibration, the ratio of the internal concentration of sodium to potassium decreased as the external ratio of potassium to sodium increased.

Lack of accumulation of chloride was accompanied by low sodium content irrespective of external concentration (Figs. 1(*a*), 1(*b*); 3(*a*), 3(*b*)) while chloride accumulation was accompanied by higher sodium content (Figs. 1(*c*) and 3(*c*)).

In the reciprocal experiments, increase of the external and internal sodium concentrations was accompanied by only small decreases of total internal potassium (Figs. 4(*a*) and 4(*b*)).

If the cations in beetroot mitochondria were partitioned between at least two phases, one a solution phase behind a semi-permeable membrane and the other a phase containing cations not participating in osmotic relations, the total cation concentration would be a poor measure of the Donnan equilibration and, consequently, sodium-potassium replacement. In Figures 5(*a*) and 5(*b*) the curves for total cations in the mitochondria deviated markedly from the Donnan expectation. It was desirable then to extract differentially those salts in solution from those not participating in the osmotic relations of the mitochondria. Consecutive extractions with aqueous HCHO and TCA were used for this purpose. Figures 5(*a*) and 5(*b*) show that the cations extracted by HCHO more closely resembled the Donnan expectation than either the cations extracted by TCA after prior extraction with HCHO or the total cations. The closer resemblance to the Donnan expectation occurred for the HCHO fractions from mitochondria whether or no they accumulated chloride.

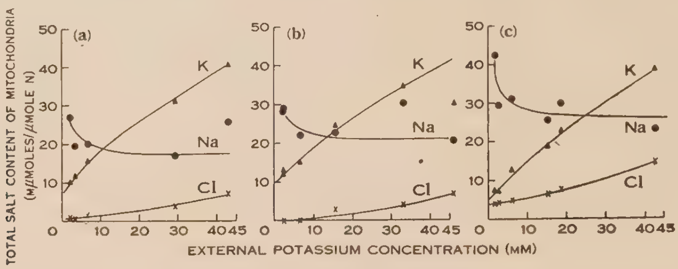

Fig. 3.—(*a*), (*b*), and (*c*) The same experiments as in Figure 1 to show the total salt content of mitochondria as functions of external potassium concentration when the added KCl only was varied.

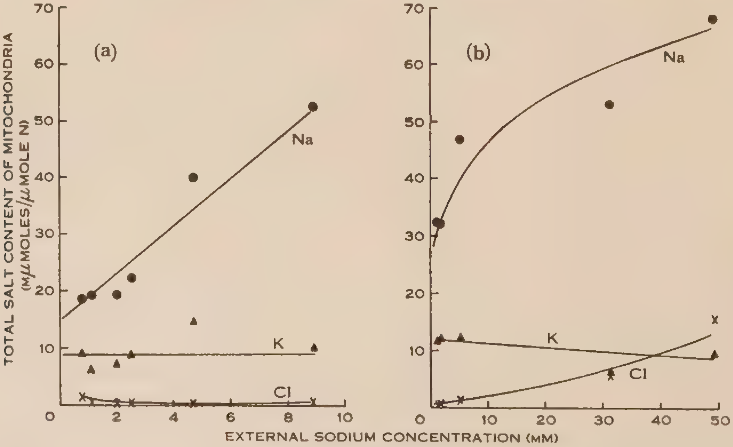

Fig. 4.—(*a*) and (*b*) The same experiments as in Figure 2 to show the total salt content of mitochondria as functions of external sodium content, when the added NaCl only was varied.

The extractions by HCHO cannot be considered to discriminate perfectly between bound ions and readily diffusible ions. For example, HCHO may be expected to increase the anionic species of proteins and thereby to promote cation binding. HCHO extraction would then result in a low estimate of the salts participating in the Donnan equilibration. Table 1 shows the results of extracting the mitochondria for total salts with TCA and for salts in solution with either water after heat denaturation or with HCHO. The results show that those cations extracted by water followed the Donnan expectation less closely than those extracted by HCHO, but the amounts of cations extracted by water exceeded those extracted by HCHO.

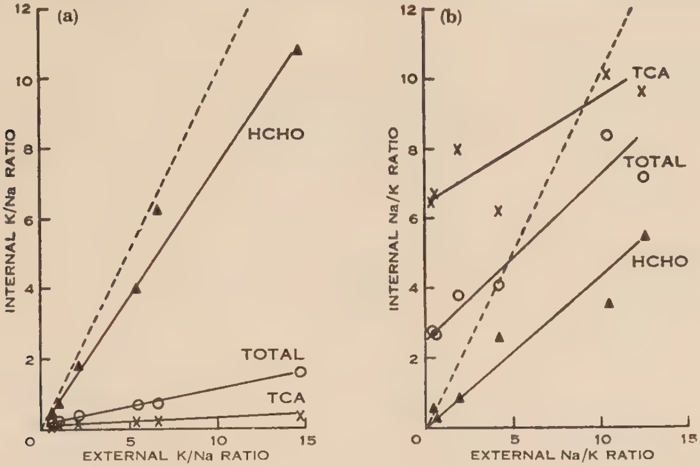

Fig. 5.—(*a*) The potassium-sodium ratios of internal and external concentrations of mitochondrial systems in KCl-fortified systems and (*b*) in NaCl-fortified systems. ---- The Donnan expectation at equilibrium. Same experiments as for Figures 1 and 2.

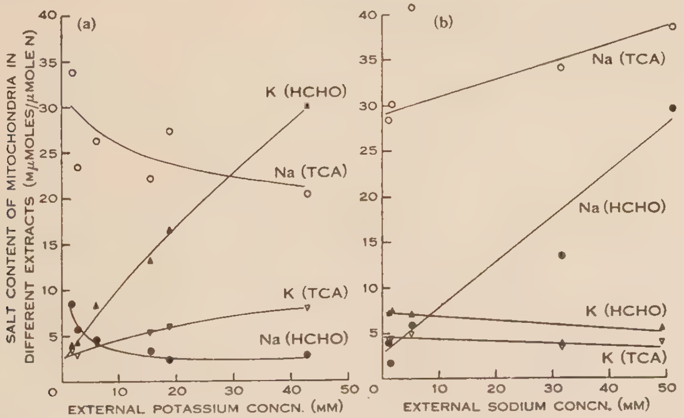

Fig. 6.—(*a*) Salt content of mitochondria in different extract fractions as functions of external potassium concentration when added KCl only was varied, and (*b*) of external sodium concentration when added NaCl only was varied.

Figures 6(*a*) and 6(*b*) show that the cations extracted by HCHO were more influenced by external concentrations than were the remaining cations extracted by

TCA. Both the sodium and potassium of the HCHO fractions were more replaceable by potassium and sodium, respectively, than were the cations extracted by TCA. Again, the effect of external potassium on internal sodium was greater than that of external sodium on internal potassium. Potassium was remarkably unaffected by external sodium level.

TABLE 1

PARTITIONING OF CATIONS IN MITOCHONDRIAL SUSPENSIONS DETERMINED BY DIFFERENT METHODS OF EXTRACTION

Experiment	A		B	
Extraction method	Water*	Total†	HCHO‡	Total†
$[K^+]_o$ (mM)	18	18	19	19
$[K^+]_{i_{50}}$ (mM)	38	40	20	54
$[Na^+]_o$ (mM)	2	2	3	3
$[Na^+]_{i_{50}}$ (mM)	17	44	3	77
$[K^+]_o/[Na^+]_o$	9·0	9·0	6·3	6·3
$[K^+]_{i_{50}}/[Na^+]_{i_{50}}$	2·1	0·9	6·7	0·7
Per cent. deviation (internal from external ratios)	−62	−81	+6	−89

* Mitochondria denatured by heating in water. Resulting suspension filtered and the filtrate used for analyses (Robertson *et al.* 1955).

† Mitochondria denatured and extracted with aqueous TCA. Suspension cleared by centrifugation and the supernatant used for analyses.

‡ Same procedure as for "total" but HCHO used in place of TCA.

(*d*) *Ion Balance of Mitochondria*

The contents of mitochondria extracted from beetroot under different conditions and incubated in different salt media are shown in Table 2. The data indicate that the salt content of the beetroot cells contributing to the external salts of the incubation medium influenced the mitochondria. The salt content for the beetroot varied in an unpredictable way, especially shown by the phosphate. The dry weight content of the mitochondria was also variable. Some of the variability in dry weight may have been due to variation in the amount of fragmented starch grains carried down with the mitochondria. The nitrogen content appeared to increase from June to September 1955, taking into account the amount of tissue extracted.

Table 2 shows that the HCHO fraction was high in chloride but low in calcium compared with the TCA fraction. Irrespective of the external concentration, the sodium content of the TCA fraction was high compared with the potassium content.

High calcium concentration of media results in mitochondria revealing an apparent adenosine triphosphatase (ATP-ase) (Witter, Watson, and Cottone 1955). Since the ATP-ase activity is a characteristic of treatments tending to disorganize mitochondria, the presence of calcium in the extraction medium for beetroot mitochondria was considered as a possible factor in decreasing their intactness. The metal-chelator EDTA was incorporated into the extraction medium as a means of lowering the effective calcium concentration. Table 2 shows that the internal calcium content was lowered with EDTA about 10–100 times, but no marked effects were found on the ability of the mitochondria to accumulate chloride under the particular conditions employed. Table 3 shows the constitution of beetroot mitochondria compared with cat-heart sarcosomes.

Table 2

ANALYSES OF MITOCHONDRIA EXTRACTED FROM BEETROOT WITH SUCROSE OR WITH SUCROSE AND ETHYLENEDIAMINETETRA-ACETATE AND INCUBATED IN SALT MEDIA AT 25°C

Mitochondria isolated from beetroot in 0·6M sucrose or 0·6M sucrose+0·136M TRIS+0·100M EDTA and resuspended in 0·6M sucrose (HB 33 and 34); or 0·6M sucrose+exogenous KCl+NaCl, 0·005M each (HB 31 and 32). Amounts of beetroot extracted were 400 g for HB 31 and HB 32, 440 g for HB 33, and 880 g for HB 34

Experiment No.	HB 31	HB 32	HB 34	HB 33
Isolation medium	Sucrose	EDTA	Sucrose	EDTA
Date	27.vi.55	7.vii.55	29.ix.55	20.vii.55
Homogenate pH	5·8	6·8	5·8	6·8
Incubation time (min)	32	33	51	83
Nitrogen (μmoles/pellet)	88·4	140	365	163
Dry wt. (μg/μmole N)	235	80·0	120	197
H_2O(μmoles/μmole N)	46·5	21·0	21·5	29·5
Total phosphate (mμmoles/μmole N)	20·9	49·1	83·3	50·0
$[\text{Phosphate}]_0$ (mM)	4·2	1·1	4·4	1·5
$[Ca^{++}]_0$ (mM)	0·8	0·4	0·1	0·5
$[Ca^{++}]_{i_{50}}$ (mM) (HCHO extract)	0·0	4·0	7·0	0·0
$[Ca^{++}]_{i_{50}}$ (mM) (TCA extract)	404	25·5	375	3·5
$[K^+]_0$ (mM)	8·3	10·9	2·4	19·8
$[K^+]_{i_{50}}$ (mM) (HCHO extract)	32·5	20·7	9·7	30·8
$[K^+]_{i_{50}}$ (mM) (TCA extract)	8·4	3·9	3·6	2·3
$[Na^+]_0$ (mM)	10·3	7·0	12·8	1·1
$[Na^+]_{i_{50}}$ (mM) (HCHO extract)	4·5	36·8	22·2	0·0
$[Na^+]_{i_{50}}$ (mM) (TCA extract)	11·8	18·2	58·8	10·1
$[Cl^-]_0$ (mM)	9·8	9·5	1·0	13·0
$[Cl^-]_{i_{50}}$ (mM) (HCHO extract)	6·3	5·7	2·2	12·0
$[Cl^-]_{i_{50}}$ (mM) (TCA extract)	1·3	0·0	2·8	1·2

(e) *The pH of Mitochondrial Isolation*

Experiments with the addition of the metal-chelator EDTA to the mitochondrial isolation medium were complicated by the associated changes of pH. Figures

7(*a*), 7(*b*), 7(*c*), and 7(*d*) show the internal salt concentrations and respiration of mitochondria isolated in media of different pH but constant EDTA concentration and incubated in a standard salt medium containing buffer to insure uniform pH.

TABLE 3

COMPARISON OF THE CONSTITUTION OF PARTICLES FROM ANIMAL AND PLANT TISSUES

Particles	Cat-heart Sarcosomes*	Beetroot Mitochondria**	
Isolation medium	EDTA	Sucrose	EDTA
[Phosphate]$_0$ (mM)	—	4·4	1·5
Acid-soluble phosphate (μmole/mg protein)	0·139	—	—
Total phosphate (μmole/mg protein)	—	0·952	0·571
[Cl$^-$]$_0$ (mM)	—	1·0	13·0
Cl (μmole/mg protein)	<0·02	0·026	0·077
[Ca^{++}]$_0$ (mM)	—	0·1	0·5
Ca (μmole/mg protein)	0·099	1·55	0·011
[K$^+$]$_0$ (mM)	—	2·4	19·8
K (μmole/mg protein)	0·15	0·079	0·189
[Na$^+$]$_0$ (mM)	—	12·8	1·1
Na (μmole/mg protein)	0·05	0·336	0·029
Mg (μmole/mg protein)	0·29	—	—

* Cat-heart sarcosomes prepared in sucrose and "Versene", unspecified concentrations (Cleland, reported in Lindberg and Ernster 1954).

** Beetroot mitochondria prepared in 0·6M sucrose and 0·6M sucrose + 0·100M EDTA + 0·136M TRIS. Resuspended in 0·6M sucrose. Values computed on the basis of the conversion factor 6·25 × nitrogen = protein.

Table 4 shows that although the concentration of chloride was not significantly correlated with pH, both sodium and potassium were significantly correlated. The concentration of sodium was influenced by pH to a greater extent than was potassium. The respiration of the mitochondria was not significantly correlated with the pH of mitochondrial isolation.

IV. DISCUSSION

(*a*) *The Donnan Equilibration*

The data for mitochondrial suspensions in KCl and NaCl show a superficial resemblance to Donnan relationships. It would be unlikely for mitochondrial systems retaining any degree of *in vivo* behaviour to show pure Donnan characteristics which are equilibrium states. It is assumed that the more intact mitochondria are those that exhibit the more pronounced departure from the Donnan equilibrium, most readily shown by chloride when it is being accumulated.

The retention of the dynamic behaviour (accumulation) as opposed to the trend to the equilibrium state (Donnan system) was accompanied by a relatively low endogenous respiration which was unaffected by variations of external salt concentration. It was shown previously (Robertson *et al.* 1955) that when chloride accumulation occurred, endogenous respiration was highly correlated with the concentration

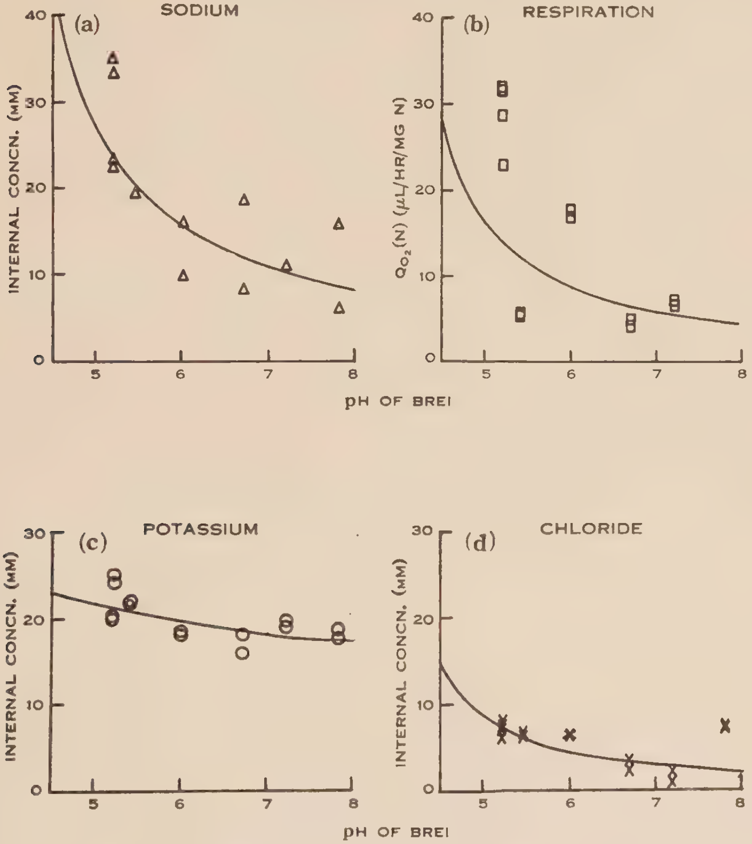

Fig. 7.—Respiration rates and internal salt concentrations of EDTA-treated mitochondria as a function of the pH of preparation. Mitochondria were extracted from beetroot with 0·4M sucrose + 10^{-2}M EDTA, pH adjusted with TRIS, and incubated in 0·4M sucrose + 10^{-2}M NaCl + 5×10^{-3}M KCl + 10^{-3}M phosphate buffer, pH 7. Lines of best fit given for $1/Y = a + bX$, calculated by least squares method.

difference maintained by the mitochondria. When exogenous substrate was added, the correlation between respiration and accumulation was not significant. Exogenous substrate may induce one-step oxidation which may be interpreted as leading to disorganization of the mitochondria. High endogenous respiration may also be indicative of some degree of disorganization (Goodwin and Waygood 1954).

(*b*) *Potassium-Sodium Replacement*

In the equilibrium state a two-phase Donnan system should show in both phases an equality of the ratios of the concentrations of any two mobile cations, e.g.

$$[Na^+]_0/[K^+]_0 = [Na^+]_i/[K^+]_i.$$

Consequently, changing the ratio of sodium to potassium in the external medium should change the ratio correspondingly in the mitochondria if their concentrations were not maintained by active processes. Both for active and inactive mitochondria, determined by the criterion of chloride accumulation, the ratios for total extractable cations deviated from the Donnan expectation. It follows that sodium and potassium contents may have been actively maintained separately from chloride maintenance or that quantities of cations were not acting osmotically, i.e. they were "bound". The latter is certainly expected to some extent since proteins form salt complexes (Cohn and Edsall 1943: see also Hope 1956).

TABLE 4

CORRELATION OF INTERNAL SALT CONTENT AND RESPIRATION RATE WITH THE pH OF MITOCHONDRIAL ISOLATION (DATA OF FIG. 7)

Quantities correlated with pH of isolation*	$1/[Cl^-]_{i_{50}}$	$1/[K^+]_{i_{50}}$	$1/[Na^+]_{i_{50}}$	$1/Q_{O_2}(N)$
Coeff. of correlation *r*	$0\cdot36 \pm 0\cdot25$	$0\cdot63 \pm 0\cdot15$**	$0\cdot68 \pm 0\cdot16$**	$0\cdot48 \pm 0\cdot22$
Coeff. of regression *b*	$0\cdot11 \pm 0\cdot06$	$0\cdot004 \pm 0\cdot001$***	$0\cdot028 \pm 0\cdot008$***	$0\cdot05 \pm 0\cdot02$
Intercept *a*	$-0\cdot42$	0·02	$-0\cdot11$	$-0\cdot21$
Number of cases	14	14	12	12

* For the relation $1/[Cl^-]_{i_{50}} = a + b(\text{pH})$, calculated by the method of least squares. All values corrected for number of cases.

** Significant at $P < 0\cdot01$.

*** Significantly different at $P < 0\cdot01$.

The exchangeable cations probably consist of the osmotically active cations in equilibrium with the bound cations as determined by mass action, pH, specificity of binding sites, stability constants, etc. As seen from Figure 6, at near zero concentration of external sodium and potassium, some exchangeable sodium remains in the mitochondria, presumably in equilibrium with the low external sodium and the high content of bound sodium in the mitochondria. This exchangeable sodium can be replaced by increasing the external potassium concentration. However, at near zero concentration of external sodium and potassium, external sodium does not replace internal potassium, presumably since little or no exchangeable potassium remains in the mitochondria owing in part to the low bound potassium content.

It was desirable to distinguish between osmotically active and inactive cations. Since many of the inactive cations were probably bound to structural components of the mitochondria differential extraction was required which would not remove structural elements. This required the extracting medium to be aqueous and without marked distorting effects on the mitochondria. Aqueous HCHO was used. Its fixation of mitochondria results in the shape and size of the mitochondria which resemble the mitochondria *in situ* (Harman and Kitiyakara 1955; Witter, Watson, and Cottone 1955).

Although the HCHO extraction was not expected to yield perfect discrimination, the cations extracted by HCHO more closely followed the Donnan expectation than either total salts or those extracted with TCA after HCHO extraction. A comparison with the extraction of mitochondria with water after heat denaturation showed that HCHO extracted less sodium and potassium. This may be interpreted as HCHO promoting cation binding which may result from an increase of anionic species of proteins owing to HCHO combination with free amino groups. However, the comparison was not completely satisfactory since in the method of water extraction (Robertson *et al.* 1955) considerable amounts of suspended mitochondria were present in the solutions analyzed and could have contributed quantities of bound cations to the analyses for osmotically active cations. This seems likely since the HCHO extracted cations more closely followed the Donnan expectation.

The adjustment of internal sodium-potassium contents as a function of external potassium concentration showed that sodium in both HCHO and TCA fractions decreased with increasing potassium concentration. On the other hand, although potassium in the HCHO fraction was decreased more by increasing external sodium than was potassium in the TCA fraction, both were relatively little affected. However, it is quite clear that both sodium and potassium of the HCHO fraction were more influenced by external concentrations.

The difference between the behaviour of the sodium and potassium of beetroot mitochondria is another example of biological systems differentiating between these two superficially similar elements. The observations agree qualitatively with those of Stanbury and Mudge (1953) for the irreplaceability of potassium by sodium in rabbit liver mitochondria.

(*c*) *Ion Balance*

Extracted beet mitochondria do not always maintain chloride in higher concentration than that in the external solution. If a Donnan system only were operating, the internal chloride (mobile anion) would be expected to be lower in concentration than the external chloride, due to the high concentration of immobile internal anions which balance the cations. Small amounts of accumulation may therefore occur if the chloride concentration exceeds the Donnan expectation, even though it may be less than that in the external solution. The previous report of active chloride accumulation (Robertson *et al.* 1955) embodied data from a relatively brief period of the year. A definite seasonal trend has been detected in the ability of mitochondria to maintain differences between internal and external chloride concentrations. The favourable period corresponded with that of the experiments previously reported. However, in periods when observed accumulation was rare, it was detected occasionally especially at low external chloride concentration. Analyses of the beetroot mitochondria for various contents did not reveal any definite trend with season, except for nitrogen, or consistent difference as a function of extraction treatment. These results emphasize the variability of the mitochondrial preparations.

Lowering of the calcium concentrations by the addition of chelating agents improves the stabilization of oxidative phosphorylation by sarcosomes (Slater and Cleland 1952) and diminishes the sarcosome permeability to solutes (Cleland 1952). Since marked permeability to ions would preclude the demonstration of chloride accumulation in mitochondria, the metal-chelator EDTA was used to promote mitochondrial stability. Mitochondria extracted from beetroot with EDTA possessed markedly lower calcium contents but did not show improved ability to accumulate chloride under the particular conditions. It was not determined whether EDTA prevented entry of calcium into the mitochondria or removed calcium previously present.

The constitution of cat-heart sarcosomes and beetroot mitochondria with respect to several elements are of the same magnitude. However, data on the constitution of particles are of limited use without the composition of the media from which the particles were isolated.

(*d*) *The pH of Mitochondrial Isolation*

The pre-treatment pH, i.e. pH during isolation of the mitochondria, had little significant effect on respiration and chloride level. These experiments uniformly showed no chloride accumulation but rather a Donnan exclusion of chloride. The sodium and potassium contents, however, increased with decrease in pre-treatment pH and were significantly different from each other as a function of pH.

The increase in both sodium content and respiration rate with decreasing pH may be related phenomena whereas the relatively uniform potassium contents may reflect a different, more stable phenomenon. If the high and variable endogenous respiration were indicative of disorganization and sodium were the major mobile cation (potassium being bound, for example, to organic polyphosphates (Melchior 1954)), then high sodium content may indicate entry of sodium into partially disorganized mitochondria to balance immobile mitochondrial anions. The lowering of pre-treatment pH may for some unexplained reason increase the immobile anions. Alternatively, low pH may impair active sodium transport, while at higher pH there may be active excretions of sodium from the mitochondria resulting in the lower internal than external sodium concentrations. Increased sodium contents and increased respiration rates may then be signs of disorganized mitochondria. Since, simultaneously, chloride was not accumulated, the possibility arises that in beetroot mitochondria there may be at least two entirely separate active transport mechanisms. It would appear that accumulation of chloride by mitochondria is an especially sensitive process indicating the intactness of the mitochondria necessary to carry out physiological work.

(*e*) *Mitochondria and Ionic Balance within Cells*

The present work has shown that the mitochondria can maintain high concentrations of internal cations, probably largely due to a Donnan adjustment, but that also some of the cations are not readily replaceable. Within the intact cell the mitochondria are likely to be local centres concentrating these cations. However, at all times the concentration within the mitochondria will be a function of the metabolic activity, which influences not only the concentrations of immobile anions but also the degree of organization of the particles.

V. Acknowledgments

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VI. References

Bartley, W., and Davies, R. E. (1954).—Active transport of ions by sub-cellular particles. *Biochem. J.* **57**: 37–49.

Cleland, K. W. (1952).—Permeability of isolated rat heart sarcosomes. *Nature* **170**: 497–8.

Cohn, E. J., and Edsall, J. T. (1943).—"Proteins, Amino Acids and Peptides." (Reinhold Publ. Corp.: New York.)

Farrant, J. L., Potter, C., Robertson, R. N., and Wilkins, M. J. (1956).—The morphology of red beet mitochondria. *Aust. J. Bot.* **4** : 117-24.

Goodwin, B. C., and Waygood, E. R. (1954).—Succinoxidase inactivation by a lecithinase in barley seedlings. *Nature* **174**: 517–8.

Harman, J. W., and Kitiyakara, A. (1955).—Studies on mitochondria. VI. The relationship between the structure, osmotic reactivity and ATP-ase activity of mitochondria from pigeon skeletal muscle. *Exp. Cell Res.* **8**: 411–35.

Hope, A. B. (1956).—The electric properties of plant cell membranes. I. The electric capacitance of suspensions of mitochondria, chloroplasts, and *Chlorella* sp. *Aust. J. Biol. Sci.* **9**: 53–66.

Lindberg, L. V., and Ernster, L. (1954).—Chemistry and physiology of mitochondria and microsomes. In "Protoplasmatologia Handbuch der Protoplasmaforschung." Vol. III. Part A4. (Springer-Verlag: Wien.)

Lowry, O. H., and Lopez, J. A. (1945).—The determination of inorganic phosphate in the presence of labile phosphate ester. *J. Biol. Chem.* **162**: 421–8.

Macfarlane, M. G., and Spencer, A. G. (1953).—Changes in water, sodium and potassium content of rat-liver mitochondria during metabolism. *Biochem. J.* **54**: 569–75.

Melchior, M. C. (1954).—Sodium and potassium complexes of ATP. Equilibrium studies. *J. Biol. Chem.* **208**: 615–27.

Mercer, F. V., Hodge, A. J., Hope, A. B., and McLean, J. D. (1955).—The structure and swelling properties of *Nitella* chloroplasts. *Aust. J. Biol. Sci.* **8**: 1–18.

Robertson, R. N. (1951).—Mechanism of absorption and transport of inorganic nutrients in plants. *Annu. Rev. Pl. Physiol.* **2**: 1–24.

Robertson, R. N. (1956).—The uptake of minerals. In "Handbuch der Pflanzenphysiologie." Vol. 4. Part IV B. (Ed. E. Ruhland; sub-ed. G. Michael.) (Springer-Verlag: Heidelberg.) (in press).

Robertson, R. N., Wilkins, M. J., Hope, A. B., and Nestel, L. (1955).—Studies in the metabolism of cells. X. Respiratory activity and ionic relations of plant mitochondria. *Aust. J. Biol. Sci.* **8**: 164–85.

Slater, E. C., and Cleland, K. W. (1952).—Stabilization of oxidative phosphorylation in heart-muscle sarcosomes. *Nature* **170**: 118–9.

SPECTOR, W. G. (1953).—Eletrolyte flux in isolated mitochondria. *Proc. Roy. Soc.* B **141**: 268–79.

STANBURY, S. W., and MUDGE, G. H. (1953).—Potassium metabolism of liver mitochondria. *Proc. Soc. Exp. Biol., N. Y.* **82**: 675–81.

STEINBACH, H. B. (1951).—Permeability. *Annu. Rev. Pl. Physiol.* **2**: 323–42.

WITTER, R. F., WATSON, M. L., and COTTONE, M. A. (1955).—Morphology and ATP-ase of isolated mitochondria. *J. Biophys. Biochem. Cytology* **1**: 127–38.

THE GENETICAL CONTROL OF INCOMPATIBILITY IN *PHALARIS COERULESCENS* DESF.

By D. L. Hayman*

[*Manuscript received February* 7, 1956]

Summary

Phalaris coerulescens Desf. is a completely self-incompatible, diploid, perennial grass. Incompatible pollen grains germinate normally but the pollen tubes fail to penetrate far into the style. The incompatibility interrelationships of parents and progenies were determined by observing the pollen tubes and pollen grains after pollination. This could be done successfully only in plants induced to flower early by exposure to long day treatment. Incompatibility is controlled by two loci, each with a series of multiple alleles. The loci are probably not linked but the data would not allow detection of loose linkage. Pollen determination is gametophytic, and the genes act independently in the style. The relationship of this incompatibility system to previously described systems is considered, and theoretically possible incompatibility systems based on genetical control at two loci are suggested.

I. Introduction

Self-incompatible flowering plants, while possessing normal male and female gametes, fail to form zygotes after self-pollination, and also after cross-pollination with certain other plants of the same species. Sterile pollinations occur if the combinations of the pollen and style are incompatible. The effect of an incompatibility system is to divide a population into a number of intra-sterile, inter-fertile, mating groups. The operation of such a system of outbreeding usually depends upon a genetically specified pollen grain being recognized as compatible or incompatible by a genetically specified style.

Analyses of the genetical control of incompatibility in the angiosperms have demonstrated the existence of two major systems. Those species of angiosperms in which the mating groups correspond to morphologically distinct groups, have an incompatibility system controlled by one or two loci each with two alleles. Dominance exists between the alleles in the style, and the specificity of the pollen is determined by the dominant gene in the diploid tissue, i.e. sporophytically. This is "heteromorphic" incompatibility (Lewis 1949*b*). Those self-incompatible angiosperms which have apparently no morphological characters corresponding to the mating groups display "homomorphic" incompatibility (Lewis 1949*b*). The majority of these possess an incompatibility system controlled by a single locus with a large number of alleles. No dominance exists between the alleles which act independently in the style, and the specificity of the pollen is determined by the allele it carries, i.e. gametophytically. A modification of this system has recently been reported in a number of composites and crucifers and in *Theobroma cacao* L. (Bateman 1954*a*, 1955; Crowe 1954; Knight and Rogers 1955). Again a single gene with a large number of alleles is concerned, but dominance differences occur between the alleles and the

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specificity of the pollen is determined by the parental genotype, i.e. sporophytically. The alleles may exhibit dominance or independence of action in the style.

Incompatibility in the Gramineae is well known, and appears to be common (Beddows 1931; Smith 1944). However, until recently no genetic analysis of the incompatibility system in a grass has been published. Lundqvist (1954, 1955) has reported that *Secale cereale* L. and *Festuca pratensis* Huds. both possess a novel system of genetical control of incompatibility. In these species incompatibility is under the control of two apparently independently-segregating loci, each with a series of multiple alleles. There is no dominance between the alleles which act independently in the style, and the specificity of the pollen is determined gametophytically.

This paper describes the independent discovery of an identical incompatibility system in *Phalaris coerulescens* Desf. (*P. aquatica* L.).

II. Materials

P. coerulescens, a native of the Mediterranean region, is a self-incompatible perennial grass with a somatic chromosome number of 14. Three plants A, B, and C, taken from the field at the Waite Agricultural Research Institute, Adelaide, were found to be inter-fertile and the crosses A×C and A×B provided the progenies initially studied. These plants were self-incompatible even when self-pollinated at the bud stage or late in the season. Meiosis was normal in all plants and virtually no aborted pollen was produced.

Table 1

THE RATIO OF COMPATIBLE TO INCOMPATIBLE POLLEN GRAINS OBTAINED FROM A NUMBER OF CROSS-POLLINATIONS BETWEEN THE PARENTS A, B, AND C

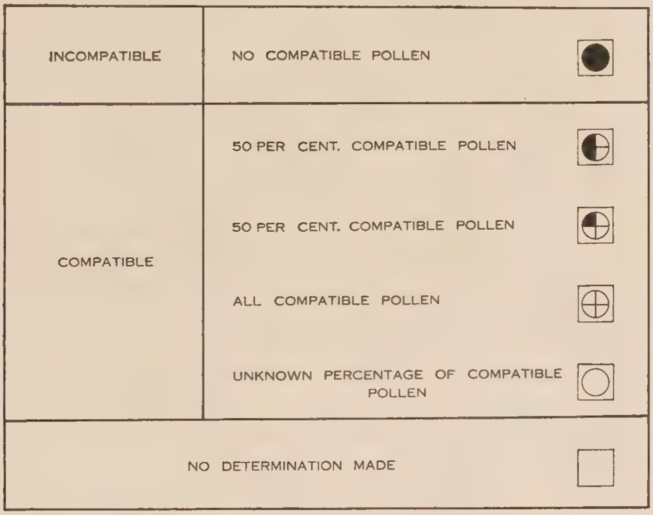
A×C	C×A	A×B	B×A	B×C	C×B
0·83	0·68	2·4	2·5	2·3	2·9
0·62	0·88	2·7	1·6	2·1	2·2
0·89	0·58	2·7	2·5	2·0	2·1
0·75	0·80	2·4	2·3	2·5	2·8
0·74	1·10	3·1		2·3	2·0
0·88	0·80	2·4		2·5	
	0·88				

III. Pollen Tube Behaviour in Compatible and Incompatible Pollinations

The compatibility or incompatibility of any cross may be gauged by the success or failure to obtain seed. Emerson (1938) showed that compatible and incompatible pollinations in *Oenothera organensis* Munz. ex Emerson could be distinguished by observing the behaviour of the pollen tubes shortly after pollination. This criterion has many advantages over that of seed-setting, and the possibility of using it in determining the compatibility of crosses in *P. coerulescens* was investigated.

The florets of *P. coerulescens* are arranged in a compact, spike-like panicle. When the plants flower at the normal time of the year (October) the period between anther exertion and dehiscence is very short and many florets in one panicle flower on the same day. Careful emasculation and pollination of a number of florets is impossible. When exposed to an artificial day length of 20 hr, the plants flower in July. Under these conditions the period between anther exertion and dehiscence is lengthened, and fewer florets on the one panicle flower simultaneously. It is therefore possible to make a number of pollinations each day. This treatment produced no abnormal effects upon the compatibility status of any crosses, or upon the amount of aborted pollen produced by any plant. The size of the panicles was reduced advantageously, but a number of sterile culms was borne by each plant. These are presumably a result of the out-of-season conditions, because they were absent when the same plants flowered later in the year.

Emasculated florets were pollinated, bagged, and collected 20 hr later. The stigmas were dissected out and placed on a slide in a drop of cotton blue in lactophenol (Watkins 1931); they were gently warmed before being covered with a cover glass.



INCOMPATIBLE	NO COMPATIBLE POLLEN	●
COMPATIBLE	50 PER CENT. COMPATIBLE POLLEN	◐
	50 PER CENT. COMPATIBLE POLLEN	◔
	ALL COMPATIBLE POLLEN	⊕
	UNKNOWN PERCENTAGE OF COMPATIBLE POLLEN	○
NO DETERMINATION MADE		□

Fig. 1.—Key to the symbols used to designate the compatibility status of a cross.

Germination of the pollen grains takes place within 5 min of pollination, and there is no difference between the germination percentage in compatible and incompatible crosses. After germination, the pollen tubes of compatible pollen grains penetrate the stigma at the nearest point and grow towards the ovary. The cytoplasmic contents of the pollen grain pass into the pollen tube shortly after germination, and the empty pollen grain stains very faintly. The pollen tubes of incompatible pollen grains usually have no difficulty in entering the stigma but do not grow very far into the style. The cytoplasm of such pollen grains is not all released into the pollen tube, and both the pollen tube and pollen grain stain very darkly (Plate 1, Fig. 1). An occasional incompatible pollen tube is twisted about the stigmatic branch before entering, and some pollen tubes are seen growing up the style instead of down it (Plate 1, Fig. 2).

Where both compatible and incompatible pollen are present on the same stigma, the ends of the incompatible pollen tubes are frequently swollen (Plate 2), and dark-staining areas are present in the style. These latter are presumably the result of the bursting of the incompatible pollen tubes and the liberation of their cytoplasm into the style. The abnormalities are present 24 hr after pollination and have only been observed when both compatible and incompatible pollen grains are present. The swelling and bursting of incompatible pollen tubes has been described in other species (Sears 1937), but in these the simultaneous presence of compatible pollen grains is not necessary.

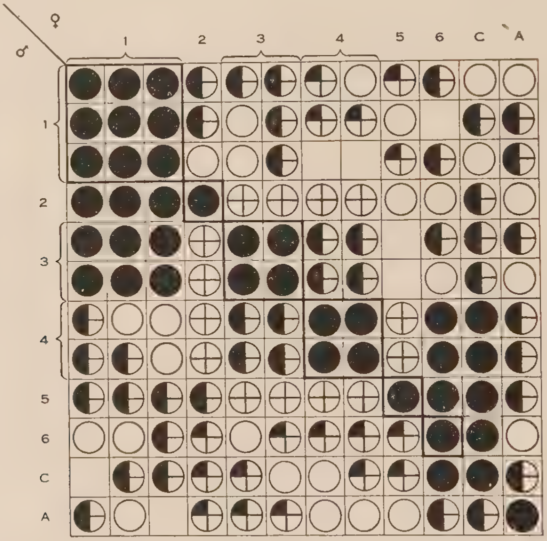


Fig. 2.—Incompatibility interrelationships of the progeny from the cross A × C, when incorporated with their parents in a diallel.

In *Secale cereale* the amount of germination of incompatible pollen increases, the further such pollen grains are from the base of the style (Sears 1937). This is not so in *Phalaris*.

An analysis of the genetical control of incompatibility was made using the pollen grain appearance as an index of compatibility, and making the crosses between plants induced to flower early. Initially, many of the results obtained were confirmed by seed-setting, but as correspondence was always exact this check was later omitted.

IV. Genetical Analysis

A number of progeny from each cross, selected at random, were intercrossed with each other and with their parent in a diallel. The crosses were scored both for their compatibility and for the ratio of compatible to incompatible pollen grains. The estimation of this ratio was hampered by the large amount of pollen shed from each anther, which causes difficulty in discriminating between incompatible pollen grains and dark-staining, ungerminated, pollen grains. Even when care is taken, the stigma is frequently smothered with pollen grains. This reduces the amount of pollen grain germination, and tends to conceal the dark-staining pollen tubes.

Depending upon the distribution and the numbers of pollen grains on a stigma, varying ratios of compatible to incompatible pollen grains may be obtained from repeated scoring of the one cross. Table 1 lists the ratios observed from several pollinations between the three parental plants. The ratios show considerable variation because of the misclassification of ungerminated pollen grains, but in the crosses A × C and C × A they approach a maximum of 1:1, or 50 per cent. compatible pollen, and in the crosses A × B, B × A, B × C, and C × B they approach a maximum of 3:1, or 75 per cent. compatible pollen.

In order to present the results concisely a series of symbols suggested to me by Dr. M. J. D. White has been adopted. They are illustrated in Figure 1.

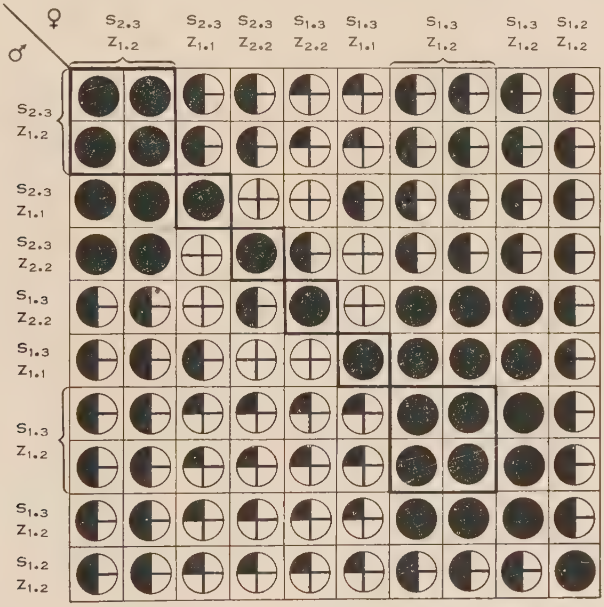


Fig. 3.—Theoretical incompatibility interrelationships of the progeny from the cross $S_{1.2}Z_{1.2} \times S_{1.3}Z_{1.2}$ when incorporated with their parents in a diallel. The relative frequencies of the genotypes are indicated.

The detailed results from the crosses A × C and A × B are given in Figures 2 and 4. More than four classes appear in each of the progenies and one of these classes is identical in behaviour with the male parental genotype. There are frequent differences in reciprocal crosses and complete incompatibility does not occur between the progeny and the female parent. Further, there are three classes of compatible pollinations. The combination of all these features cannot be explained by the operation of a single gene with multiple alleles. To explain these results the following theory was postulated. The genetical control of incompatibility in *P. coerulescens* is due to two independently-segregating loci, each with a series of multiple alleles. The genes act independently in specifying the compatibility behaviour of the style, and the specificity of the pollen grain is determined gametophytically. A pollen grain is incompatible on a style when the two genes it carries are both present in that style.

The letters chosen to designate the loci and their alleles are $S_{1....n}$ and $Z_{1....n^1}$, where n and n^1 may or may not be equal. These letters have been used by Lawrence (1930) and adopted by Lundqvist (1954); to introduce a new notation would only add confusion.

Genotypes which would give a 1:1 ratio of compatible to incompatible pollen, when reciprocally crossed, are either $S_{1.1}Z_{1.2} \times S_{1.2}Z_{1.1}$ or $S_{1.2}Z_{1.2} \times S_{1.3}Z_{1.2}$. The first of these alternatives would result in only two classes among the progeny. The expected interrelationships when the progeny from the second cross are incorporated with their parents in a diallel are given in Figure 3. It is apparent that these expectations are fully realized by the progeny from the cross A×C (see Fig. 2).

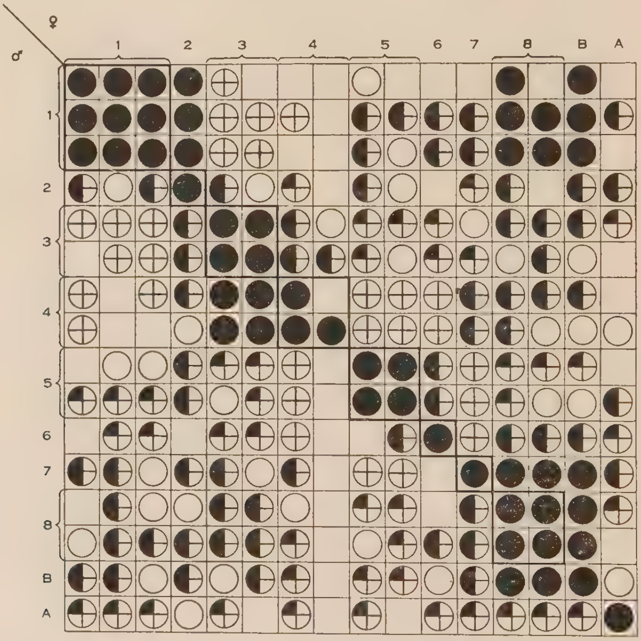


Fig. 4.—Incompatibility interrelationships of the progeny from the cross A×B, when incorporated with their parents in a diallel.

Genotypes which would yield a 3:1 ratio of compatible to incompatible pollen grains when reciprocally crossed are $S_{1.2}Z_{1.2} \times S_{2.3}Z_{1.3}$. The expected behaviour of the progeny of this cross when incorporated in a diallel with their parents is given in Figure 5. The eight observed classes in the cross A×B have been allocated to eight of the theoretical genotypes in Figure 5.

While the hypothesis adequately explains the data, the results from a further cross prove its validity. If the genotypes allocated to the classes 7 and 8 in the cross A×B are correct, then the behaviour of the progeny from this cross, when incorporated with their parents in a diallel, would be as illustrated in Figure 6. The actual results obtained with the progeny from this cross are given in Figure 7. There is complete agreement between the expected and observed results in all respects.

Close linkage between the two loci is most unlikely. Unfortunately the number of plants tested is too low to detect loose linkage between the *S* and *Z* loci. Linkage tests would be easier if marker genes linked to either of the incompatibility loci were found. Such linkage in the appropriate crosses would result in a deviation from normal Mendelian ratios in the progeny, but strict testing would be required to distinguish this from certation. The two single gene controlled mutants so far identified in *P. coerulescens* show no evidence of linkage to either of the incompatibility loci.

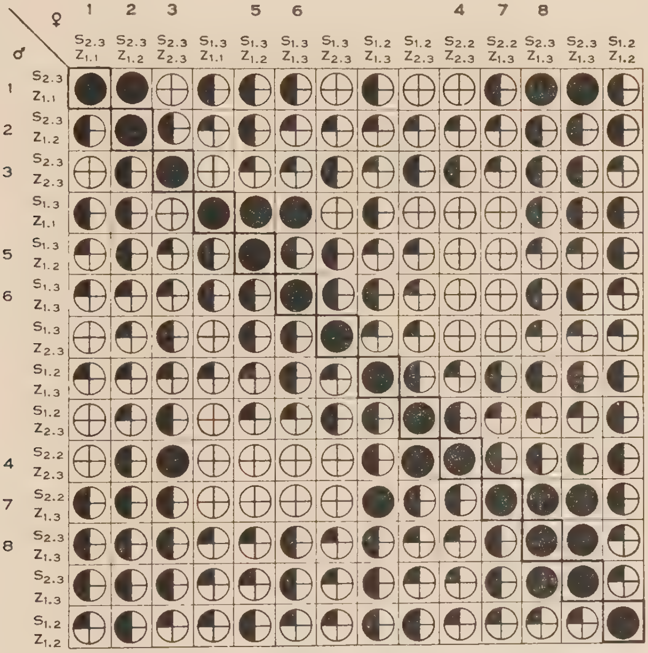


Fig. 5.—Theoretical incompatibility interrelationships of the progeny from the cross $S_{1.2}Z_{1.2} \times S_{2.3}Z_{1.3}$ when incorporated with their parents in a diallel. The incompatibility classes in Figure 4 are allocated to the appropriate genotypes in this figure.

During the experiments no self-fertile plants were observed but two seeds were obtained from selfed heads. These seeds did not produce self-fertile plants, and their relationship to their parent is under investigation.

V. Discussion

Williams (1951) has suggested that entemophilous species are more likely to evolve a multiple allelomorph type of incompatibility than anemophilous species. He argues that compulsory insect visitation ensures both "self" and "foreign" pollen being present on the stigmas, and considers that in anemophilous species it is possible for self pollen to reach the stigma some time before foreign pollen. Thus it is possible that insect pollination provides a better mechanism for the selective elimination of self pollen than does wind pollination. However, these arguments cannot be applied to the Gramineae. Grasses usually grow together in large populations. In those species morphologically adapted to out-pollination, in which anthesis takes place external to the glumes, large amounts of pollen are shed. Both self and foreign pollen may therefore arrive simultaneously on a stigma. Under these conditions an opportunity exists for the selective elimination of self pollen, and the development of the multiple allelomorph type of incompatibility.

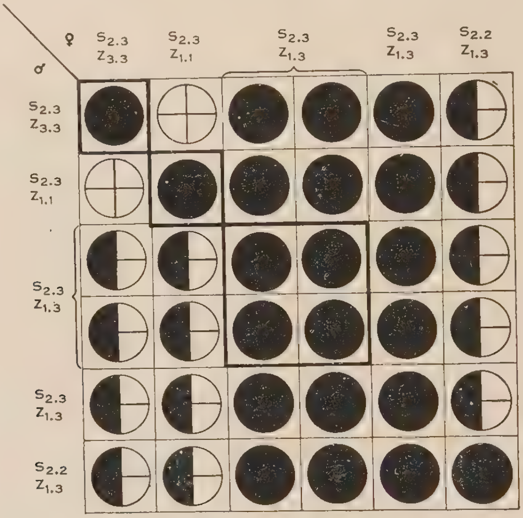


Fig. 6.—Theoretical incompatibility interrelationships of the progeny from the cross $S_{2.2}Z_{1.3} \times S_{2.3}Z_{1.3}$ when incorporated with their parents in a diallel.

The evidence available from previous genetical analyses of incompatibility in the angiosperms suggests that all plants in the same taxonomic family have the same system of genetic control. The evidence from the identity of the incompatibility systems in *Secale*, *Festuca*, and *Phalaris* supports this. In addition, Adams (1953) has reported differences in the compatibility of reciprocal crosses in *Bromus inermis* Leyss. and mentions other instances. Such differences occur in both the two-loci system with gametophytic determination, and in the single-locus sporophytic system. Further work on the genetical control of incompatibility in the Gramineae is required. It has frequently been shown that the self-incompatibility of the majority of grasses is not absolute, some seeds being obtained from self-fertilization. This may be explained by environmental factors, variation in the efficiency of the incompatibility sieve for different incompatibility alleles, or to the action of genetic modifiers. More precise observations of the compatibility of crosses would be obtained by using the appearance of the pollen grains as an indication, rather than the occurrence of seed-setting.

Bateman (1954*b*) has listed the differences between the single-locus gametophytic and sporophytic systems found in the angiosperms. The essential diagnostic difference between these systems and the "Gramineae" system, is in the number of possible inter-fertile, intra-sterile classes which are possible in the progeny of a cross. There may be two, three, six, 12, or 16 classes, depending upon the genotypes of the parents.

The single-locus gametophytic system enforces out-breeding while not completely restricting matings between sister plants. More restriction on sister-plant matings results from the occurrence of dominance differences between the alleles as in the sporophytic systems. The incorporation of a second locus, with a series of allelic genes, into the genetical control of incompatibility allows a higher frequency of outbreeding between both sister and non-sister plants, and conserves alleles more successfully against loss by random drift (Lundqvist 1954).

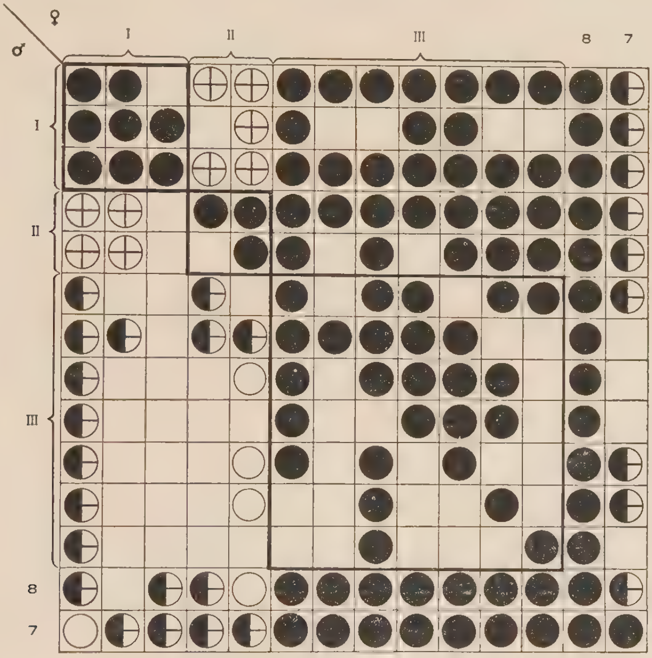


Fig. 7.—Incompatibility interrelationships of the progeny from the cross 7 × 8 when incorporated with their parents in a diallel.

There is a resemblance between the genetical control of incompatibility in the fungi and in the angiosperms. The three systems (genetical control by a single locus with two alleles, genetical control by multiple alleles of single locus, and genetical control by multiple alleles of two unlinked loci) are found in both groups of plants. However, the correspondence is not complete since dominance differences do not occur in the haploid fungi, and there is no counterpart in the fungi to genetical control by two loci each with two alleles. Further, in the two-loci system of the fungi, incompatibility occurs when the thalli concerned have either one or both of the incompatibility genes in common.

Papazian (1951) has presented evidence which shows that the two incompatibility loci in *Schizophyllum commune* Fries. are probably compound loci consisting of a number of closely-linked genes. A similar explanation of the multiple alleles in

Phalaris and *Secale* is possible, although no evidence of such a situation has been found in *Oenothera*, even after stringent tests (Lewis 1948, 1949*a*, 1951).

Further modifications of the genetical control of incompatibility systems by segregation of genes at a single locus are impossible, since all conceivable variations have been reported. However, it is possible that variations of the two-loci system will be found. Such variations may result in dominance differences in either or both of the allelic series or in an incompatible pollen grain needing to possess only one allele in common with the style. This latter would resemble the situation found in some heterogenic pollen grains of autotetraploid *Oenothera organensis* (Lewis 1947). It is also possible that the two loci may be linked; as a consequence of this the frequencies of the genes in the population would be unaltered but the relative frequencies of the various linked combinations would be different. Complete linkage of the two loci would make this system identical to the single-locus gemetophytic system.

Lundqvist (1954) argues that duplication of an original single-locus system is the most probable origin of the two-loci incompatibility system. The evidence from the duplicated incompatibility systems in autotetraploid *O. organensis* and autotetraploid *Trifolium repens* L. does not support this (Lewis 1947; Brewbaker 1954). Competition and dominance occur between the genes in the diploid pollen grain, and the four genes in the style act independently. As a consequence of this, the incompatibility system may break down, and self-fertility result. It is more likely that the two loci concerned with the incompatibility specification of the pollen grain were both originally present; or that one locus became concerned later, without derivation from the original locus. In this way the complementary nature of the genes' action would be present initially, and the complications consequent upon duplication avoided.

The interesting and valuable studies of Emerson (1939) and Atwood (1944) on the distribution of the incompatibility genes in natural populations are unlikely to be repeated in *Secale* and *Phalaris*. In these species incompatibility occurs between gene combinations rather than between single genes, and the number of testers required, and the difficulty in obtaining them, would discourage such an attempt.

VI. Acknowledgments

The original observation of self-incompatibility in *P. coerulescens* was made by Dr. A. T. Pugsley, who provided the author with some of the material. The study was made under the guidance of Professor D. G. Catcheside to whom the author expresses his thanks. The study was made during the tenure of a C.S.I.R.O. Studentship and a Senior Research Scholarship of the University of Adelaide.

VII. References

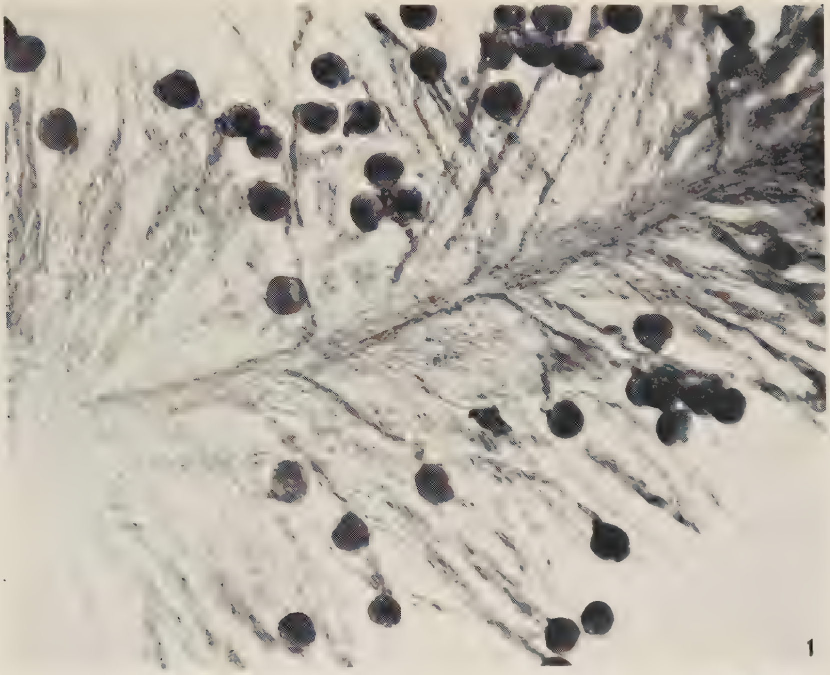
Adams, M. W. (1953).—Cross and self-incompatibility in relation to seed formation in *Bromus*. *Bot. Gaz.* **115**: 95–105.

Atwood, S. S. (1944).—Oppositional alleles in natural populations of *Trifolium repens*. *Genetics* **29**: 428–35.

Bateman, A. J. (1954*a*).—Self-incompatibility systems in angiosperms. II. *Iberis amara*. *Heredity* **8**: 305–22.

Bateman, A. J. (1954*b*).—The diversity of incompatibility systems in flowering plants. Proc. 8th Int. Bot. Congr. Paris. pp. 138–45.

INCOMPATIBILITY IN PHALARIS COERULESCENS



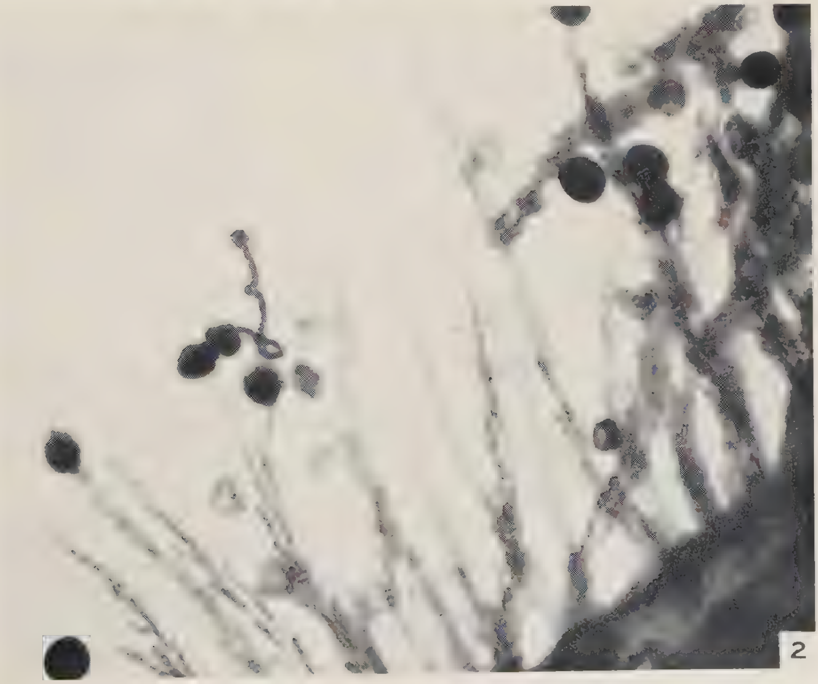
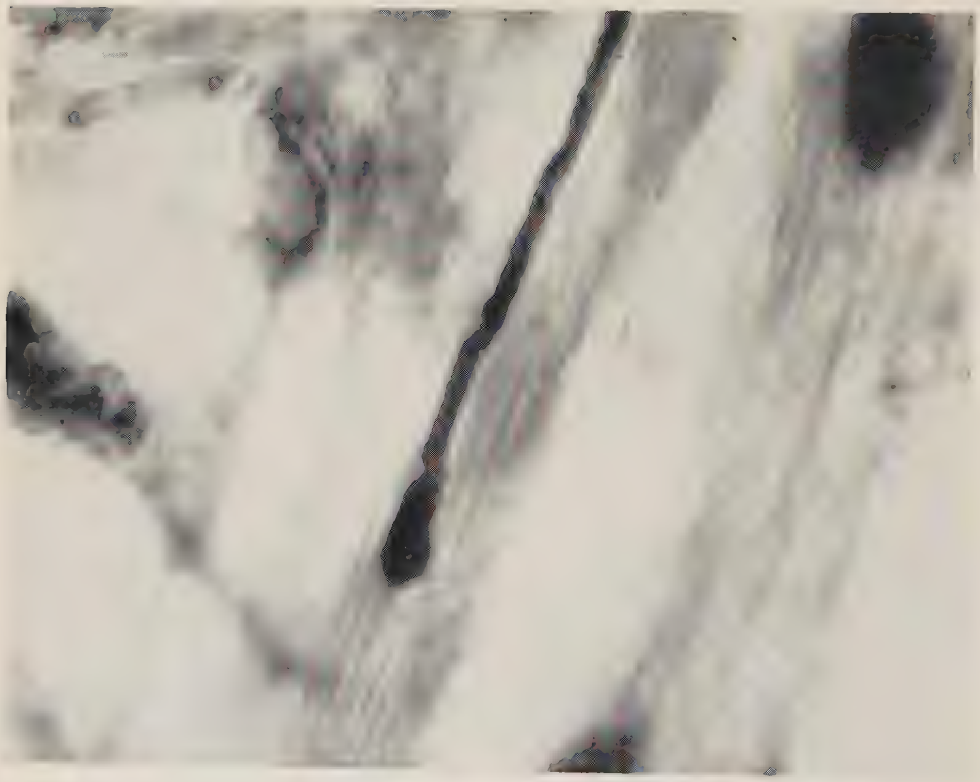


Fig. 1.—Pollen tube growth 48 hr after an incompatible pollination.

Fig. 2.—An abnormality of pollen tube growth 24 hr after pollination. The incompatible pollen tube has grown up the stigmatic hair and the end of the tube appears swollen. The faintly-staining pollen grains are compatible.

INCOMPATIBILITY IN PHALARIS COERULESCENS



The swollen end of an incompatible pollen tube in the style—24 hr after pollination.

Bateman, A. J. (1955).—Self-incompatibility systems in angiosperms. III. *Cruciferae*. *Heredity* **9**: 53–68.

Beddows, A. R. (1931).—Seed setting and flowering in various grasses. *Bull. Welsh Pl. Breed. Sta. Series H.* **12**: 79.

Brewbaker, J. L. (1954).—Incompatibility in autotetraploid *Trifolium repens* L. I. Competition and self-compatibility. *Genetics* **39**: 307–16.

Crowe, L. K. (1954).—Incompatibility in *Cosmos bipinnatus*. *Heredity* **8**: 1–11.

Emerson, S. (1938).—Genetics of self-incompatibility in *Oenothera organensis*. *Genetics* **23**: 190–202.

Emerson, S. (1939).—Preliminary survey of the *Oenothera organensis* populations. *Genetics* **24**: 524–40.

Knight, R., and Rogers, H. H. (1955).—Incompatibility in *Theobroma cacao*. *Heredity* **9**: 69–77.

Lawrence, W. J. C. (1930).—Incompatibility in polyploids. *Genetica* **12**: 269–96.

Lewis, D. (1947).—Competition and dominance of incompatibility alleles in diploid pollen. *Heredity* **1**: 85–108.

Lewis, D. (1948).—Structure of the incompatibility gene. I. Spontaneous mutation rate. *Heredity* **2**: 219–36.

Lewis, D. (1949*a*).—Structure of the incompatibility gene. II. Induced mutation rate. *Heredity* **3**: 339–55.

Lewis, D. (1949*b*).—Incompatibility in flowering plants. *Biol. Rev.* **24**: 472–96.

Lewis, D. (1951).—Structure of the incompatibility gene. III. Types of spontaneous and induced mutations. *Heredity* **5**: 399–414.

Lundqvist, A. (1954).—Studies on self-sterility in rye, *Secale cereale* L. *Hereditas, Lund.* **40**: 278–94.

Lundqvist, A. (1955).—Genetics of self-incompatibility in *Festuca pratensis*. *Hereditas, Lund.* **41**: 518–20.

Papazian, H. P. (1951).—The incompatibility factors and a related gene in *Schizophyllum commune*. *Genetics* **36**: 441–52.

Sears, E. R. (1937).—Cytological phenomena connected with self-sterility in the flowering plants. *Genetics* **22**: 130–81.

Smith, D. C. (1944).—Pollination and seed formation in grasses. *J. Agric. Res.* **68**: 79–95.

Watkins, A. E. (1931).—Genetical and cytological studies in wheat. *J. Genet.* **15**: 323–66.

Williams, W. (1951).—Genetics of incompatibility in alsike clover (*T. hybridum*). *Heredity* **5**: 51–73.

FACTORS INFLUENCING THE DEVELOPMENT OF BROWN PATCH IN LAWNS OF *SAGINA PROCUMBENS* L.

By A. Kerr*

[*Manuscript received January* 24, 1956]

Summary

The meteorological conditions necessary for the appearance of brown patch caused by *Pellicularia praticola* (Kotila) Flentje in *Sagina* lawns in Adelaide have been determined. They are:

(i) A minimum temperature between 63·5 and 68°F, and

(ii) Free moisture or very high humidity at the lawn surface.

The time necessary for infection to take place was determined at one temperature only (77°F) and was 8–12 hr.

Results indicate that the causal fungus is able to grow as a saprophyte beneath the surface of a well-watered lawn, but that intensive saprophytic growth is dependent on an adequate nutrient supply which, under natural conditions, is provided by a previous period of parasitic growth.

I. Introduction

Brown patch disease of lawns was first described by Piper and Coe (1919) who isolated the casual organism and determined it as *Rhizoctonia solani* Kühn (*Pellicularia filamentosa* (Pat.) Rogers). This fungus has a wide host range and may infect many lawn grasses (Howard, Rowell, and Keil 1951). In South Australia, *Sagina procumbens* L. (pearlwort) has been commercialized as a lawn plant, its chief merit being that it does not require cutting. It has been planted in several gardens in Adelaide and has proved very susceptible to brown patch. The disease occurs in two forms: firstly, as more or less circular patches of dead lawn, 6–12 in. in diameter, or secondly, it may appear as "smoke rings", also more or less circular, but only those plants in a strip 2–3 in. wide round the perimeter of the ring are killed (Plate 1, Fig. 1). A smoke ring may be 1–8 ft in diameter or even larger. Where the disease is serious, strips of dead lawn from several patches may meet and it is then difficult to recognize their circular nature. Plants killed by the fungus are at first dark green, almost black in colour, due to the water-soaked character of the infected tissues which later dry out and become light brown.

The influence of environmental conditions on brown patch has been noted by several workers who, in general, agree that the appearance of the disease is dependent on warm, moist weather, but who disagree as to the relative importance of temperature and humidity on it. Dickinson (1930) stated temperature was the important factor, humidity influencing the disease only by its effect on air and surface soil temperature. He defined the optimum temperature conditions for the development of brown patch as:

(i) A temperature of 64–68°F for 45 min to stimulate germination of resting sclerotia, followed by

(ii) A rise in temperature to between 80 and 85°F.

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Dahl (1933) did not agree with the findings of Dickinson. Working with several strains of the fungus he found that the minimum temperature for germination of sclerotia was between 46 and 54°F, and the optimum temperature between 82 and 90°F. The sclerotia did not require any drop in temperature to stimulate germination. Monteith and Dahl (1932) stated that the conditions most favourable for the development of brown patch are a temperature between 80 and 90°F and "the presence of ample water". Because of the conflicting evidence on the exact conditions which favour the occurrence of brown patch, the appearance of the disease in *Sagina* lawns in Adelaide has been studied in relation to weather conditions.

The fungus used in the present work was Waite Institute strain St 3 which was isolated from a smoke ring in a *Sagina* lawn. It differs from most isolates of *P. filamentosa* from other hosts in having a faster rate of growth and a typical white mealy appearance in culture (potato-dextrose-agar); it has been designated *P. praticola* by Flentje (1956).

II. EXPERIMENTAL

(*a*) *The Relation between the Occurrence of Brown Patch in* Sagina *Lawns in Adelaide and Weather Conditions*

Brown patch in a *Sagina* lawn was first recorded in Adelaide on February 11, 1952. During the following summer (1952–53) 12 lawns within a radius of 3 miles of the Waite Institute were visited regularly, and the appearance of patches in these lawns was noted. The days on which patches appeared are given in Table 1, together with the Waite Institute meteorological records for the 24 hr ending 9 a.m. on these days. The weather in each instance was warm or hot, and rain was recorded on each occasion except one, when only a single lawn was affected. Enquiries were made

TABLE 1

DAYS ON WHICH BROWN PATCH APPEARED AND THE METEOROLOGICAL RECORDS FOR THE 24 HR ENDING 9 A.M. ON THESE DAYS

Days on which Brown Patch Appeared	No. of Lawns Affected	Air Temp.		Soil Temp. (1-in. depth)		Rainfall (in.)	R.H. (at 9 a.m.)
		Max.	Min.	Max.	Min.		
11.ii.52	?	72·8	57·8	93·6	58·2	0·160	68
15.xi.52	2	85·3	61·2	70·5	58·5	0·265	86
5.xii.52	2	74·2	60·1	99·5	61·5	0·075	66
26.xii.52	8	78·6	58·2	107·2	62·3	0·040	54
5.i.53	4	93·2	59·5	81	66	0·390	95
25.i.53	9	95·1	67·8	98	65·8	1·0	80
26.i.53		92·5	68·8	107·2	67·2	0·1	50
27.i.53		90·7	76·4	101·0	72·8	0·025	64
5.ii.53	7	87·0	61·2	82	61·3	0·495	79
16.iv.53	1	91·2	73·5	87·3	64·5	Lawn watered	43

and it was found that that lawn had been watered on the evening before the patches appeared.

This suggested that free water was necessary for the appearance of brown patch, and to test this the parasitic activity of the casual fungus, with and without free moisture present, was studied in the laboratory. Constant relative humidity was maintained in a growth cabinet by means of an automatic humidifier controlled by a cellulose acetate–mercury hygrometer (Ballard 1949). A relative humidity of 93 per cent. was maintained with a variation of ± 2 per cent. R.H. Six inoculated and six control plugs of a *Sagina* lawn, 3 in. in diameter, were sprayed with water, placed in 4-in. diameter glass jars, and transferred to the growth cabinet at a temperature of 77°F. Half of the jars were covered with plastic to prevent evaporation of water. After 2 days the controls at both humidities and the inoculated plugs at 93 per cent. R.H. remained healthy. In the inoculated lawns with free moisture present, the fungus had grown out radially from the inoculum and killed the lawn in a circle 2 in. in diameter. This experiment shows that a relative humidity higher than 93 per cent. is necessary for the parasitic activity of *P. praticola*, but in Adelaide there are very few days with a temperature around 77°F and an air humidity above 93 per cent. without free moisture being present on the surface of a lawn.

Table 2

TEMPERATURE AT TIME OF RAINFALL ON DAYS WHEN BROWN PATCH APPEARED

Days on which Brown Patch Appeared	Rainfall (in.)	Time of Rainfall (hr)	Air Temp. at Time of Rainfall (°F)	1-in. Depth Soil Temp. at Time of Rainfall (°F)
11.ii.52	0·160	1200–1600	—	76–75
15.xi.52	0·265	0400–0730	76–65	66–67
5.xii.52	0·075	0530	68	65
26.xii.52	0·040	1400–1600	70–72	75–78
5.i.53	0·390	0100–0900	66–61	72–66
25.i.53	1·0	1600	94–70	80
		0830	79	68
26.i.53	0·1	1700	92	90
27.i.53	0·025	1700	88	104
5.ii.53	0·495	1700–1800	80–74	81–76
16.iv.53	Lawn watered	1920–2030	83–84	80–78

If free moisture is essential for the development of brown patch, the maximum and minimum temperature for the previous 24 hr are not so important as the temperature of the lawn when the free moisture is present. The time of rainfall during the 24 hr prior to the appearance of brown patch was determined from a Julien Friez continuous rain recorder, and this is compiled with the corresponding air temperature and soil temperature at 1 in. depth in Table 2 (a continuous grass temperature record was not available). The air temperature varied between 61 and 94°F and the soil temperature between 65 and 104°F. Although temperature at time of rainfall was determined, it was not known how long free moisture remained on the lawn surface after the rain, and so a complete record could not be obtained for the period when moisture conditions were suitable for parasitism by the fungus. The evidence suggests that the minimum temperature for the appearance of brown patch is between 60 and 70°F.

For more precise information the pathogenicity of *P. praticola* to *S. procumbens* was studied in the laboratory at 59, 68, and 77°F. Three replicates of inoculated and control lawn plugs in glass jars were placed in Wisconsin tanks at these temperatures, sprayed with water, and covered with plastic. After 3 days there were diseased patches in the inoculated plugs at 68 and 77°F whereas the inoculated lawns at 59°F and all the control lawns were healthy. The six lawn plugs at 59°F were examined again after 1 week, but there was still no infection.

A further experiment was carried out, using the same methods but at temperatures of 59, 63·5, and 68°F. At 59 and 68°F the result was as before. At 63·5°F a typical patch was not formed, but the fungus had grown out and a few leaves were infected, so that this temperature must be very near the minimum at which *P. praticola* will infect *S. procumbens*, but it is too low for the fungus to cause a typical brown patch.

The infection period, or the measure of time required for the establishment of the parasitic relationship had yet to be determined. With this object in view, 4-mm discs of inoculum were floated on sterile water in Petri dishes until hyphae grew from the discs which were then carefully transferred to plugs of *Sagina* lawn. This ensured that there was no delay in the growth of the fungus after inoculation. The plugs were sprayed with water and covered with plastic. Three plugs were removed after 4, 8, 12, 16, and 24 hr and carefully examined for infection. Owing to scarcity of material the infection period could be determined only at one temperature, 77°F. Occasional infection was observed after 8 hr and infection was common after 12 hr.

It can be stated, therefore, that the conditions of temperature, moisture, and time necessary for the appearance of brown patch in a *Sagina* lawn are:

(i) A minimum temperature between 63·5 and 68°F,
(ii) Free moisture or very high humidity at the lawn surface, and
(iii) An infection period of 8–12 hr at the one temperature tested (77°F).

(*b*) *Saprophytic Activities of the Brown Patch Fungus*

The character of a smoke ring suggests that the initial growth of the fungus is saprophytic because the centre of the patch remains healthy. To test this, two circular plugs of *S. procumbens* lawn, 10 in. in diameter, were inoculated at the centre and half an inch below the surface of the lawn with 1 g of sand-maize-meal inoculum of *P. praticola* and placed in a growth room at 72°F and watered regularly. After 7 days there was no sign of parasitic activity and the extent of saprophytic growth of the fungus was determined by planting on to tap-water-agar pieces of lawn plants taken from below the surface and at half-inch intervals along four radii from each plug. *P. praticola* had grown to the edge of the plugs.

The result of this experiment indicates that the fungus is capable of extensive saprophytic growth beneath the surface of a well-watered lawn where there is always free moisture or a very high humidity. When it rains, or the lawn is watered, the fungus will be able to grow to the surface and parasitize the leaves of the lawn plants if the temperature is above 63·5°F. The size of the patch will depend on the extent of saprophytic growth and the duration of conditions suitable for parasitism. If the saprophytic growth is limited a small brown patch will be formed; if extensive, a smoke ring patch will result, the width of the ring being determined by the duration of conditions suitable for parasitism.

An attempt was made to produce a smoke ring in the laboratory. A plug of *Sagina* lawn, 10 in. in diameter, was placed in a glass dish of the same diameter. The plug was inoculated at the centre and 1 cm beneath the surface, with 1 g of sand-maize-meal inoculum, and placed in a constant temperature (72°F) growth room at approximately 50 per cent. relative humidity. It was watered regularly for 8 days and the lawn showed no sign of disease. The lawn was then sprayed with water, covered with clear plastic to prevent evaporation, and examined after 24 hr. The plug of lawn was diseased except for a strip, approximately 1 in. wide, round the periphery (Plate 1, Fig. 2). A smoke ring was not formed. The result confirms the hypothesis except for the formation of the smoke ring. It is likely that the extent of saprophytic growth was not sufficient for a ring to be formed.

The factors which stimulate very dense saprophytic growth of the fungus are not yet known. It does not grow intensively during the winter because the first patches that appear in the early summer are always small, 6 in.–1 ft in diameter. As the summer advances and conditions suitable for parasitism occur, larger patches appear, and by the end of the summer patches 6–8 ft in diameter or even larger are formed. Brown patch may occur as a double ring (see Plate 1, Fig. 1), the outer ring appearing after the inner. For five such double rings from separate lawns, the radial difference between the inner and outer rings was measured, and, as the days on which these appeared were known, it was possible to calculate the rate of growth of the fungus in the lawns. The size of the rings was also known and therefore the day on which growth started could be estimated (radius of patch/rate of growth). It was calculated that in three patches growth commenced on December 6, 1952 and, in the remaining two, on December 22, 1952. Such an estimation must necessarily be approximate since it assumes that the fungus grows at a constant rate, but it is interesting to note that brown patch was recorded on December 5, 1952, and December 26, 1952 (see Table 1). This suggests that the saprophytic growth of the fungus in a patch, or in a potential patch, starts after an initial period of parasitic activity. To test this, 1 g of soil, known to be infected with *P. filamentosa*, was placed in the centre of each of two plugs of *Sagina* lawn 8 in. in diameter. The plugs were sprayed with water and one was covered with clear plastic; both were kept at a temperature of 72°F. The plastic cover was removed after 24 hr and it was seen that the leaves in the centre of the plug were parasitized. Both lawns were left for a further 6 days without covers and watered regularly. There was no sign of parasitism during this time. The lawns were then sprayed with water and covered with plastic for 24 hr, after which they were examined again. That which was infected during the initial period of 24 hr had now a diseased patch 6–7 in. in diameter, while the other had a patch approximately 1 in. in diameter. Intensive saprophytic growth appears to depend on an initial period of parasitism.

III. Discussion

From the above results the probable behaviour of the brown patch fungus, *P. praticola*, in a lawn can be described. During winter when temperatures are too low for parasitic activity the survival of the fungus must be limited, otherwise a patch formed one year would reappear the following year in the same spot, and with a diameter as great as, or greater than, that of the original patch. This does not occur and probably the fungus survives as a saprophyte or as dormant mycelium or sclerotia in isolated spots either in the lawn or in the soil beneath the lawn. In the early summer when conditions suitable for parasitism prevail, the fungus grows to the surface of the lawn, and parasitizes the leaves of the lawn plants. This initial period of parasitism provides the fungus with nutrients which enable it to grow intensively and extensively as a saprophyte beneath the lawn surface where there is always high humidity or free moisture (nutrients may be provided artificially by using high nutrient inoculum such as sand-maize-meal). When conditions suitable for parasitism again occur, the fungus will once more grow to the lawn surface and become parasitic. If the previous saprophytic growth was extensive the disease will be in the form of a smoke ring because of the greater hyphal activity at the growing edge of the colony than behind it; if the previous saprophytic growth was limited, the disease will be in the form of a brown patch. This alternating parasitic and saprophytic growth may continue throughout the summer with the result that patches formed towards the end of summer will, in general, be much larger than those formed at the beginning.

Very high humidity or free moisture is essential for the development of brown patch, its influence being direct and not indirect by its effect on temperature as stated by Dickinson (1930). This confirms Shurtleff (1953) who found that the sclerotia of *P. filamentosa* would germinate only at relative humidities of 98 per cent. or higher.

IV. Acknowledgments

I wish to thank Dr. C. G. Hansford and Dr. N. T. Flentje, under whose supervision this work was carried out, and Mr. K. Phillips for the photography.

V. References

Ballard, L. A. T. (1949).—A simple device for controlling relative humidity. *Aust. J. Sci.* **12**: 76.

Dahl, A. S. (1933).—Effect of temperature on brown patch of turf. Abstr. in *Phytopathology* **23**: 8.

Dickinson, L. S. (1930).—The effect of air temperature on the pathogenicity of *Rhizoctonia solani* parasitising grasses on putting green turf. *Phytopathology* **20**: 597.

Flentje, N. T. (1956).—Studies on the fungus *Pellicularia filamentosa* (Pat.) Rogers. I. The formation of the perfect stage by different strains. *Trans. Brit. Mycol. Soc.* **39**: (in press).

Howard, F. L., Rowell, J. B., and Keil, H. L. (1951).—Fungus diseases of turf grasses. Bull. R.I. Agric. Exp. Sta. No. 308. 56 pp.

Monteith, J., and Dahl, A. S. (1932).—Turf diseases and their control. *Bull. U.S. Golf Assoc. (Green Section)* **12**: 85.

Piper, C. V., and Coe, H. S. (1919).—*Rhizoctonia* in lawns and pastures. *Phytopathology* **9**: 89.

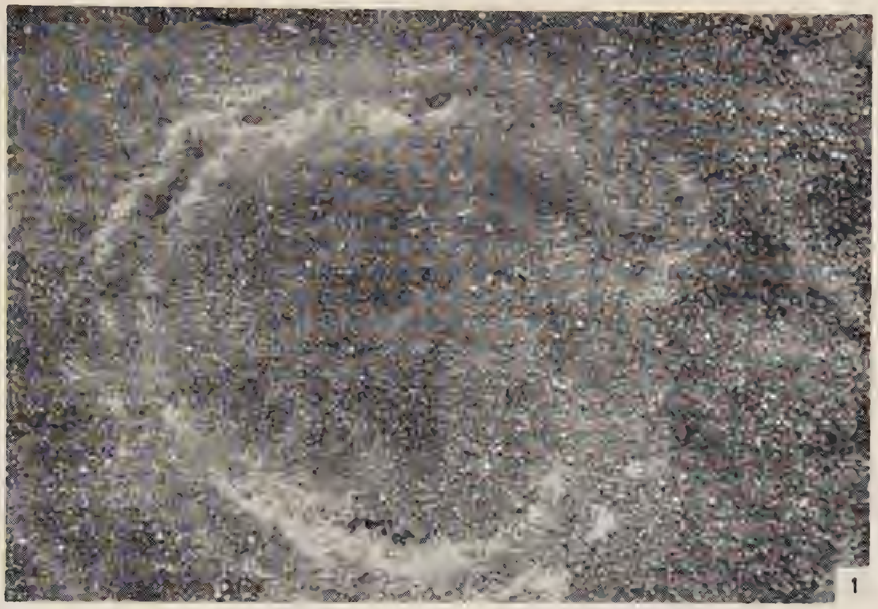
Shurtleff, M. C. (1953).—Factors that influence *Rhizoctonia solani* to incite turf brown patch. Abstr. in *Phytopathology* **43**: 484.

EXPLANATION OF PLATE 1

Fig. 1.—Brown patch in a *Sagina* lawn showing a typical smoke ring. × $\frac{1}{8}$.

Fig. 2.—Brown patch produced by *P. praticola*, after saprophytic growth for 8 days followed by parasitic growth for 1 day. × $\frac{2}{5}$.

BROWN PATCH IN SAGINA PROCUMBENS LAWNS



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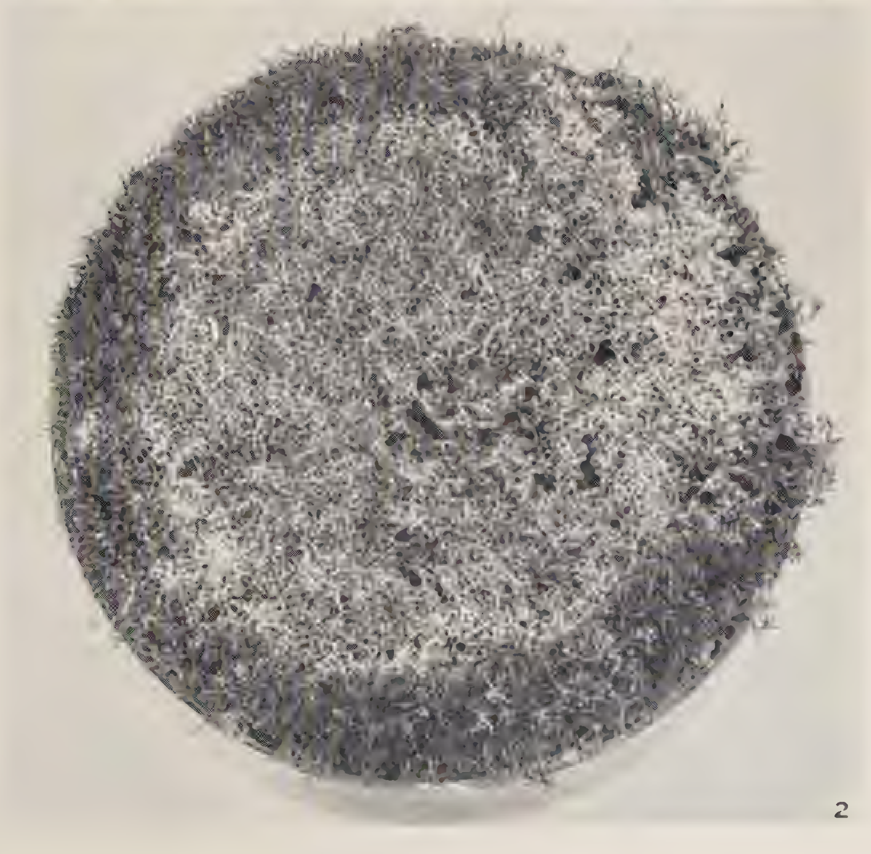

2

ENZYMIC ACTIVITIES OF SUB-CELLULAR PARTICLES FROM LEAVES

II. CENTRIFUGAL FRACTIONATION AND CHARACTERIZATION OF PARTICLES IN HOMOGENATES OF ETIOLATED PEA LEAVES

By R. M. Smillie*

[*Manuscript received December* 13, 1955]

Summary

Homogenates of etiolated pea leaves were fractionated by differential centrifugation. The fractions were examined microscopically, and were assayed for nitrogen, nucleic acid, and pigment content, for the following enzymes—succinoxidase, cytochrome oxidase, and cytochrome reductase, and for their ability to carry out oxidative phosphorylation. These studies revealed at least four different types of particles, namely nuclei, plastids, mitochondria, and particles of mitochondrial size, or smaller, lacking succinoxidase. These latter particles contained cytochrome reductase and corresponded to the microsomes of mammalian cells.

I. Introduction

The characterization of the different types of sub-cellular particles in plant cells has until recently been confined largely to cytological investigations (Newcomer 1951; Weier and Stocking 1952). In biochemical studies, particulate fractions containing mitochondrial activities have been isolated from a number of etiolated tissues (Millerd and Bonner 1953) and green leaves (Ohmura 1955; Smillie 1955*a*, 1955*b*), while the oxidation of palmitic acid by a microsomal fraction isolated from peanut cotyledons has been demonstrated by Humphreys *et al.* (1954).

Fractionation of cell-free preparations from various plant tissues by differential centrifugation has yielded further information on the nature of sub-cellular particles and their role in the metabolism of the plant cell. Fractionation procedures have been applied to etiolated pea seedlings (Stafford 1951), green tobacco leaves (McClendon 1953; Jagendorf and Wildman 1954), and potato tubers (Levitt 1954). In these investigations particles of different size were separated, but studies of their enzymic complements revealed little information about their possible function in the cell.

Very little is known of the biochemical properties of sub-cellular particles isolated from etiolated leaves. These leaves can respire, but cannot carry out photosynthesis. They possess plastids which may contain a large number of the enzymes functioning in normal photosynthesis. Hence characterization of the types of sub-cellular particles in etiolated leaf cells, and the functions of these particles as indicated by their enzymic activities, serves not only to supplement our knowledge of etiolated plant tissues in general, but by comparing such information with that from similar studies with green leaves, may also help to elucidate some of the problems regarding respiratory and photosynthetic mechanisms in green leaves. In this paper it will be shown that there exist at least four different types of sub-cellular particles in etiolated leaf homogenates. These have been fractionated by differential centrifugation.

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II. Methods

(a) *Plant Material*

Pea seedlings, *Pisum sativum* (Yates Greenfeast), were grown in darkness at 20–25°C. Etiolated leaves were harvested 10 days after germination.

(b) *Fractionation Procedure*

Etiolated leaves (20 g) were ground with sucrose (0·5M) containing ethylenediaminetetra-acetate (EDTA) (0·005M) at pH 7·2. The resulting mixture was filtered through a double layer of muslin, made up to 80 ml with sucrose-EDTA solution, and subjected to the fractionation procedure outlined in Table 1. The relative centrifugal force was calculated for the centre of gravity of the fluid volume in the centrifuge tube at each speed used. Each fraction was washed by resuspension in 80 ml of the extraction medium and re-centrifugation, and the particles were finally resuspended in sucrose (0·5M). All operations were performed at 0–5°C.

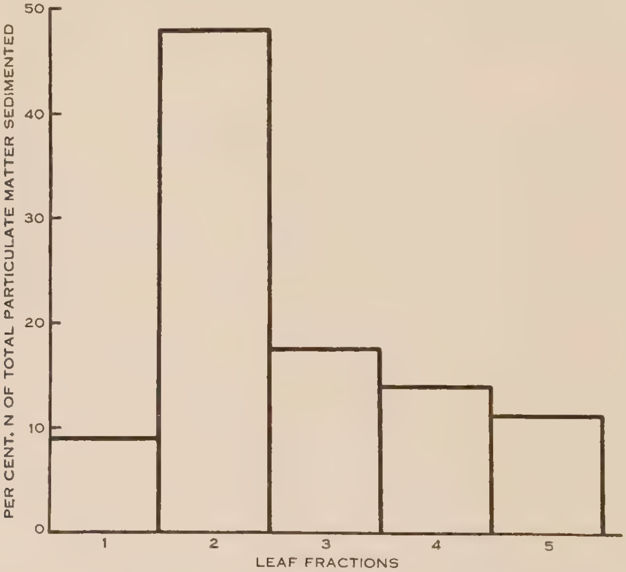

Fig. 1.—The distribution of total nitrogen in particulate fractions from etiolated leaves.

(c) *Estimations*

Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were separated by the Schmidt-Thannhauser procedure, as employed by Davidson, Frazer, and Hutchison (1951). RNA was estimated by total phosphate (Allen 1940) and DNA by total phosphate and the Dische test (Racker 1952). Carotenoids were estimated by extraction in boiling chloroform: methanol (2:1), followed by measurement of the optical density (O.D.) of the filtered extract at 449 mμ. Cytochrome oxidase was measured manometrically in the presence of exogenous cytochrome *c* and *p*-phenylenediamine. Succinoxidase, oxidative phosphorylation, and diphosphopyridine nucleotide (DPN)-linked cytochrome reductase were estimated as previously described (Smillie 1956).

III. RESULTS

(a) *Total Nitrogen Distribution in Sedimented Leaf Particles*

Figure 1 shows the distribution of total nitrogen in the five fractions isolated by sedimentation at different centrifugal forces. The highest centrifugal force used (17,000 *g*) was insufficient to sediment all the particles completely, and a clear supernatant was not obtained. The particles in the 17,000 *g* supernatant together with those lost during the washing procedure were neglected. They amounted to less than 10 per cent. of the total particulate proteins. It can be seen that almost 50 per cent. of the total particulate nitrogen was in fraction 2; the amount of material in the subsequent fractions was progressively smaller.

TABLE 1

FRACTIONATION PROCEDURE FOR HOMOGENATES OF ETIOLATED PEA LEAVES

Fraction Number	Relative Centrifugal Force (× *g*)	Time of Centrifugation (min)
1	35	5
2	1,200	15
3	2,700	30
4	6,700	15
5	17,000	45

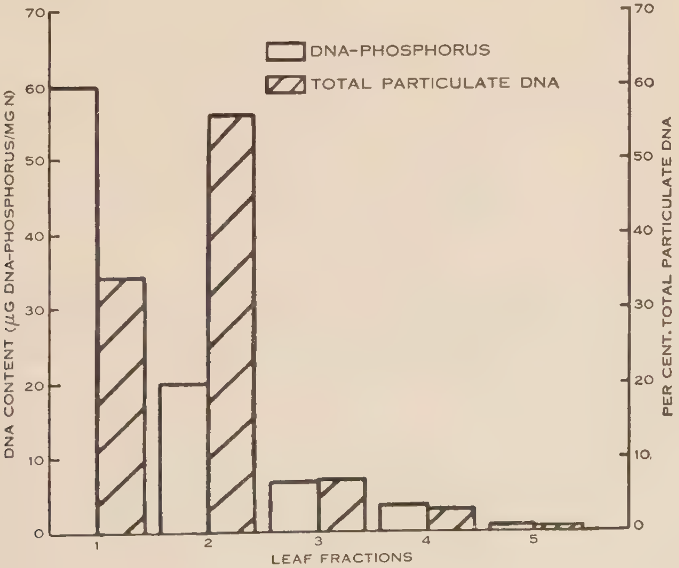

Fig. 2.—The distribution of DNA in particulate fractions from etiolated leaves.

(*b*) *Distribution of DNA and RNA*

The DNA content of the various fractions serves as a measure of the nuclei or nuclear fragments present, if it is assumed that nuclei are the only particles in normal cells which contain significant amounts of DNA. The distribution of DNA is shown in Figure 2. These results, based on the amount of deoxyribose present as indicated by the Dische test, agreed closely with values obtained by total phosphate estimation. The DNA-containing material was largely sedimented in the first two fractions. The remaining 10 per cent. present in the remaining fractions probably represents nuclear fragments produced by the grinding process. Microscopic examination disclosed many whole nuclei in fractions 1 and 2.

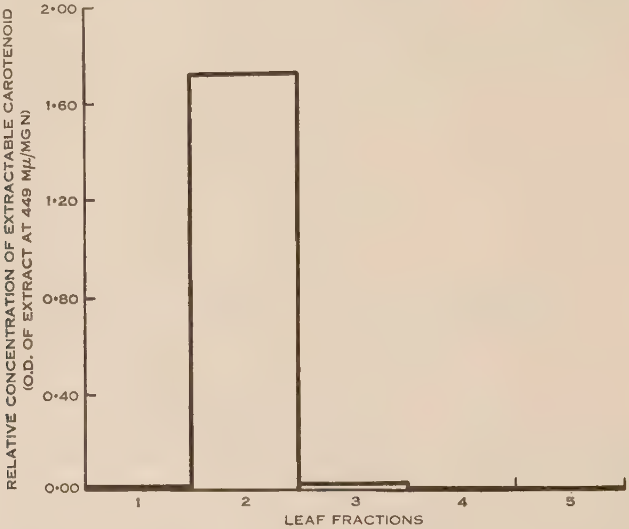

Fig. 3.—The distribution of extractable carotenoid in particulate fractions from etiolated leaves.

The RNA content, expressed on a total nitrogen basis, was highest in fraction 2. Comparison of values for both nucleic acids disclosed an RNA : DNA ratio of 0·48 for fraction 1 and 4·5 for fraction 2. The DNA content of the other fractions was too low to permit accurate estimation of this ratio.

(*c*) *Distribution of Extractable Carotenoid*

The distribution of carotenoid pigments is shown in Figure 3. Fractions 3–5, although pale yellow in colour, did not contain any significant amounts of extractable pigment. Hence it was concluded that whole plastids were almost entirely sedimented in fraction 2.

(*d*) *Distribution of Oxidative and Phosphorylative Activities*

In Figure 4 are shown the distributions of succinoxidase, and the capacity for oxidative phosphorylation in the presence of added substrate. Both activities, which are characteristic of mitochondria, had similar distribution patterns. Most of the activity was concentrated in fractions 3 and 4, isolated between 1200 and 6700 *g* respectively. It was previously shown by Smillie (1955*a*) that a similar fraction from etiolated pea leaf homogenates will also oxidize α-ketoglutarate, glutamate, *iso*citrate, citrate, and pyruvate. Etiolated leaves thus possess mitochondrial particles which are sedimented between 1200 and 6700 *g*.

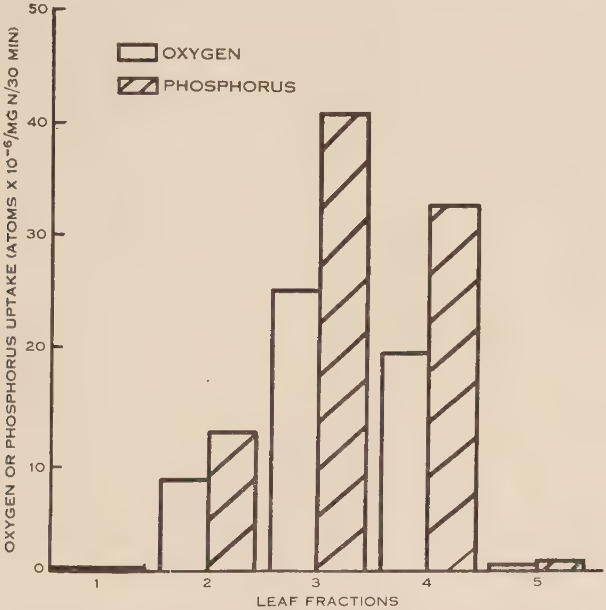

Fig. 4.—The distribution of oxidative and phosphorylative capacities of particulate fractions from etiolated leaves. Reaction mixture contained enzyme, sucrose (0·35M), potassium phosphate at pH 7·3 (0·03M), $MgSO_4$ (0·001M), adenylate (0·0005M), yeast coenzyme concentrate (1 mg), glucose (0·02M), hexokinase (0·2 mg), and succinate (0·02M). Final volume 2·0 ml.

(*e*) *Distribution of Cytochrome Oxidase and Cytochrome Reductase*

The distributions of cytochrome oxidase and DPN-linked cytochrome reductase were quite different (Fig. 5). Fractions 3 and 4 contained the highest concentrations of cytochrome oxidase. Comparison of this distribution with those of typical mitochondrial systems, such as succinoxidase and oxidative phosphorylation, indicate that cytochrome oxidase is located in the mitochondria. Similarly in etiolated pea seedlings (Stafford 1951), corn seedlings (Haskins 1955), and green tobacco leaves (McClendon 1953; Jagendorf and Wildman 1954), cytochrome oxidase was found to be localized in particles of mitochondrial size.

Little is known of cytochrome reductase in plant tissues, but in mammalian cells separations of cytoplasmic particles by differential centrifugation (Hogeboom, Schneider, and Striebich 1953) or by gradient centrifugation (Thompson and Mikuta 1954) showed that cytochrome reductase is concentrated in particles (microsomes) smaller than mitochondria. Similarly with etiolated pea leaf homogenates, the highest concentration of cytochrome reductase was found in particles smaller than mitochondria and lacking succinoxidase activity.

(*f*) *Microscopic Examination of Etiolated Leaf Fractions*

The different fractions were examined by phase-contrast microscopy. In fraction 1 the only regularly-shaped particles were whole nuclei. Fragments of cell wall were also observed. Fraction 2 contained numerous whole plastids and also many particles 1–5 μ in diameter. The other fractions contained particles varying in diameter from approximately 2 μ down to the limit of resolving power of the microscope, the average size of the particles being smaller in each succeeding fraction. In Figure 6 the distribution of particles 0·5–2 μ in diameter (corresponding to mitochondria in size) in fractions 1–4 is compared with the succinoxidase distribution. In fraction 5 there were no significant numbers of particles 1 μ or larger, all the particles being at the limit of resolution and consequently too small for accurate counting. There was no significant succinoxidase activity in this fraction.

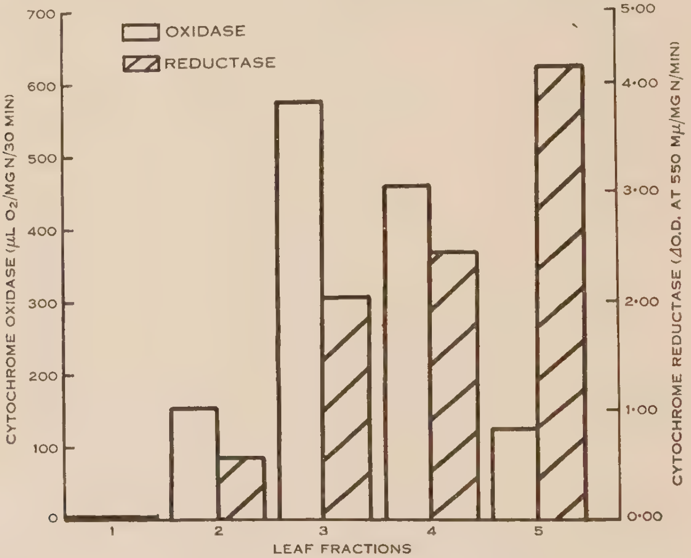

Fig. 5.—The distribution of cytochrome oxidase and DPN-linked cytochrome reductase in particulate fractions from etiolated leaves. For cytochrome oxidase assays the reaction mixture contained enzyme, potassium phosphate at pH 7·3 (0·01M), cytochrome *c* (10^{-5}M), and *p*-phenylenediamine (0·02M). Final volume 2·0 ml. For cytochrome reductase assays the reaction mixture contained enzyme, potassium phosphate at pH 7·3 (0·01M), oxidized cytochrome *c* ($5{\cdot}2\times10^{-9}$M), yeast coenzyme concentrate (1 mg), alcohol dehydrogenase (0·2 mg), KCN (0·0002M), and ethanol (0·1M). Final volume 3·0 ml.

IV. Discussion

The results obtained show that differential centrifugation of etiolated leaf homogenates successively sediments at least four different types of particles, viz. (1) nuclei, (2) plastids, (3) mitochondria, and (4) particles smaller than mitochondria (microsomes) which lack succinoxidase activity. Repeated sedimentation under a suitable gravitational force can yield preparations either of nuclei or whole plastids almost completely free of other particles. This is not so with the smaller particles.

The distribution patterns of cytochrome oxidase, succinoxidase, and oxidative phosphorylation are similar and these activities appear to reside in the same particles. Comparison with the pigment distribution and microscopic examination of the fractions show that these particles are distinct from plastids, are about 1 μ in diameter, and hence correspond to the mitochondria of other etiolated plant tissues (Millerd and Bonner 1953) and mammalian cells (Green 1954).

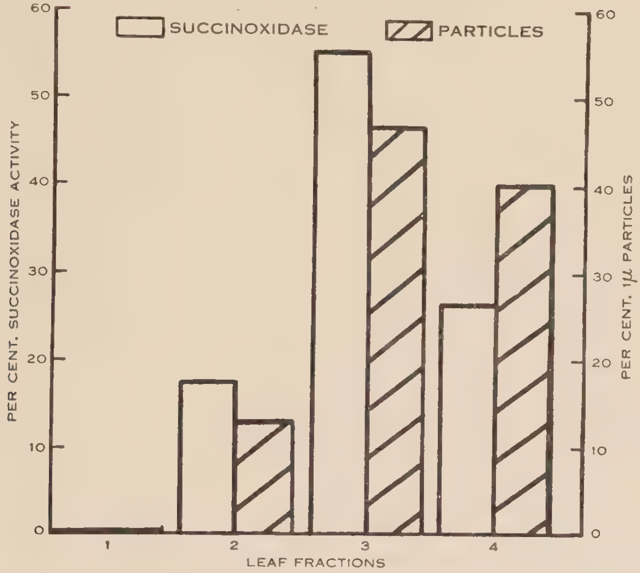

Fig. 6.—Comparison of the distribution of succinoxidase activity with the distribution of particles of mitochondrial size in particulate fractions from etiolated leaves.

The cytochrome reductase activities of the fractions containing the particles of mitochondrial size are quite high (Fig. 5) leading to the possible conclusions that either mitochondria also contain cytochrome reductase, or that smaller particles possessing this enzymic activity are adsorbed on to the larger mitochondria. A simple admixture of fractions 3 and 4 with smaller particles similar to those found in fraction 5 is unlikely, since each fraction was washed once. An alternate explanation is as follows. Since the ratio of succinoxidase activity to the number of particles about 1 μ in diameter (Fig. 6) decreases from fractions 2 to 4, it is likely that in fraction 3, and more so in fraction 4, a considerable number of particles counted do not possess succinoxidase activity and hence are similar to the smaller particles in fraction 5. The number of these particles are probably sufficient to account for the cytochrome reductase activity in fractions 2, 3, and 4. If the reductase activity in these fractions is contained in particles similar to the smaller particles of fraction 5, then it can be seen that while the average size of the latter particles are smaller than the mitochondrial particles, there is no sharp division into two different particle sizes, separable by differential centrifugation. This is in contrast to the work of Levitt (1954) who found in potato tubers no gradation in size between particles of mitochondrial and microsomal dimensions. Fractions 2–5 would also contain proplastids, but these are probably distinct from the particles described above, since whole plastids showed none of these enzymic activities.

V. ACKNOWLEDGMENTS

The author is indebted to the Commonwealth Scientific and Industrial Research Organization for financial assistance, and to the Research Committee, University of Sydney, for the purchase of chemicals. The author also wishes to thank Dr. A. Millerd for her continued interest in the course of this investigation.

VI. REFERENCES

ALLEN, R. J. L. (1940).—*Biochem. J.* **34**: 858.

DAVIDSON, J. N., FRAZER, S. C., and HUTCHISON, W. D. (1951).—*Biochem. J.* **49**: 311.

GREEN, D. E. (1954).—In "Chemical Pathways of Metabolism." Vol. 1. p. 27. (Ed. D. M. Greenberg.) (Academic Press Inc.: New York.)

HASKINS, F. A. (1955).—*Plant Physiol.* **30**: 74.

HOGEBOOM, G. H., SCHNEIDER, W. C., and STRIEBICH, M. J. (1953).—*Cancer Res.* **13**: 617.

HUMPHREYS, T. E., NEWCOMB, E. H., BOKMAN, A. H., and STUMPF, P. K. (1954).—*J. Biol. Chem.* **210**: 941.

JAGENDORF, A., and WILDMAN, S. G. (1954).—*Plant Physiol.* **29**: 270.

LEVITT, J. (1954).—*Physiol. Plant.* **7**: 109.

McCLENDON, J. H. (1953).—*Amer. J. Bot.* **40**: 260.

MILLERD, A., and BONNER, J. (1953).—*J. Histochem. Cytochem.* **1**: 254.

NEWCOMER, E. (1951).—*Bot. Rev.* **17**: 53.

OHMURA, T. (1955).—*Arch. Biochem. Biophys.* **57**: 187.

RACKER, E. (1952).—*J. Biol. Chem.* **197**: 347.

SMILLIE, R. M. (1955*a*).—*Aust. J. Biol. Sci.* **8**: 186.

SMILLIE, R. M. (1955*b*).—*Aust. J. Sci.* **17**: 217.

SMILLIE, R. M. (1956).—*Aust. J. Biol. Sci.* **9**: 81.

STAFFORD, H. A. (1951).—*Physiol. Plant.* **4**: 696.

THOMPSON, J. F., and MIKUTA, E. T. (1954).—*Arch. Biochem. Biophys.* **51**: 487.

WEIER, T. E., and STOCKING, C. R. (1952).—*Bot. Rev.* **18**: 14.

ENZYMIC ACTIVITIES OF SUB-CELLULAR PARTICLES FROM LEAVES

III. CENTRIFUGAL FRACTIONATION AND CHARACTERIZATION OF PARTICLES IN HOMOGENATES OF GREEN LEAVES

By R. M. Smillie*

[*Manuscript received December* 13, 1955]

Summary

Homogenates of green pea leaves have been fractionated by differential centrifugation. Six fractions so obtained were examined microscopically and assayed for total nitrogen, deoxyribo-, and ribonucleic acid, starch, and chlorophyll contents, succinic dehydrogenase, succinoxidase, α-ketoglutarate oxidase, cytochrome reductase, cytochrome oxidase, and their oxidative phosphorylation activity, and photolytic capacity. These studies revealed the presence of highly-significant numbers of biochemically-active mitochondria and smaller, non-oxidative particles, containing cytochrome reductase. Both particle types were distinct from chloroplasts, or chloroplast fragments. The enzyme distributions were almost identical with those found for etiolated leaves revealing a basic similarity between dark- and light-grown leaf cells.

I. Introduction

Homogenates of etiolated pea leaves were previously fractionated by differential centrifugation and the particles in these fractions have been characterized by chemical analysis and enzymic assay (Smillie 1956*b*). Similar fractionation procedures have now been applied to green pea leaves and the results compared with those for etiolated leaves. It has been shown that green leaves contain not only chloroplasts, which are the site of the photochemical fixation of carbon dioxide (Arnon, Allen, and Whatley 1954), but also particles which contain the mechanism for the respiratory oxidation of tricarboxylic acid cycle substrates to carbon dioxide and water (Smillie 1956*a*). Other enzymic activities such as cytochrome oxidase (McClendon 1953; Jagendorf and Wildman 1954), oxalic oxidase (Arnon and Whatley 1954), and catalase (Jagendorf and Wildman 1954), have already been associated with particles of mitochondrial size isolated from green leaves.

II. Methods

(*a*) *Plant Material*

Pea seedlings, *Pisum sativum* (Yates Greenfeast) were grown in a glasshouse and leaves were harvested after 2–3 weeks growth.

(*b*) *Fractionation Procedure*

Leaves (40 g) were ground in sucrose (0·5M) containing ethylenediaminetetra-acetate (EDTA) (0·005M) at pH 7·2. The mixture was filtered through a double layer

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of muslin and the filtrate made up to 80 ml with the sucrose-EDTA solution. This filtrate was then fractionated according to the procedure in Table 1 to give six fractions. Each fraction was washed once with 80 ml of the sucrose-EDTA solution and finally resuspended in sucrose (0·5M). All operations were performed at 0–5°C.

Table 1

FRACTIONATION PROCEDURE FOR HOMOGENATES OF GREEN LEAVES

Fraction	Relative Centrifugal Force ($\times g$)	Time of Centrifugation (min)
1	35	5
2	300	15
3	1,200	15
4	2,700	30
5	6,700	15
6	17,000	45

(c) *Estimations*

Starch was estimated as reducing sugar after acid hydrolysis, the sucrose first being removed by repeated washing of the fractions with alcoholic trichloroacetic acid. Chlorophyll was determined by the method of Arnon (1949), and photochemical capacity, using ferricyanide as the oxidant, as described by Arnon and Whatley (1949). Procedures for the other estimations have already been described (Smillie 1956*a*, 1956*b*).

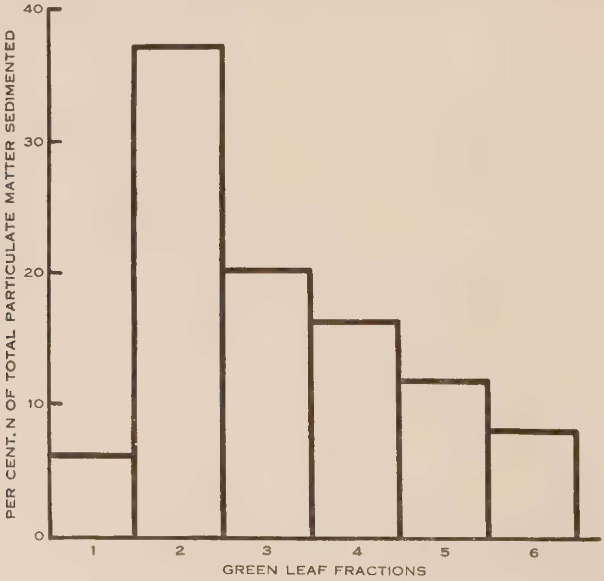

Fig. 1.—The distribution of total nitrogen in particulate fractions from green leaves.

III. Results

(a) *Distribution of Total Nitrogen*

The distribution of total nitrogen is shown in Figure 1. 30–40 per cent. of the total nitrogen was found in fraction 2, and progressively smaller amounts in the following fractions.

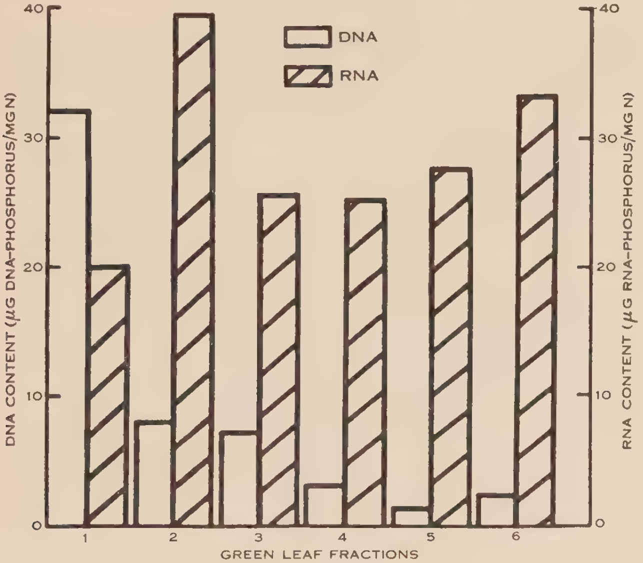

Fig. 2.—The distributions of DNA and RNA in particulate fractions from green leaves.

(b) *Distribution of Nucleic Acid*

The distributions of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are illustrated in Figure 2. Fraction 1 contained the highest concentration of DNA per mg nitrogen. The small amounts in fractions 3–6 can probably be attributed to the presence of fragments of nuclei disrupted during the grinding. RNA was present in all fractions, the largest concentration being in fraction 2. Results for RNA based on pentose determinations (Drury 1948) gave the same distribution pattern. The ratio of RNA : DNA for fractions 1, 2, and 3 were 0·62, 4·9, and 3·6 respectively. In previous studies with etiolated leaves, similar distributions of the nucleic acids were obtained (Smillie 1956*b*), values for RNA : DNA ratio of 0·48 and 4·5 being found for fractions corresponding to green leaf fractions 1 and 2 plus 3 combined respectively.

Table 2

THE DISTRIBUTION OF STARCH IN PARTICULATE FRACTIONS FROM GREEN LEAVES

Fraction	1	2	3	4	5	6
Total starch (%)	89	9	2	0	0	0

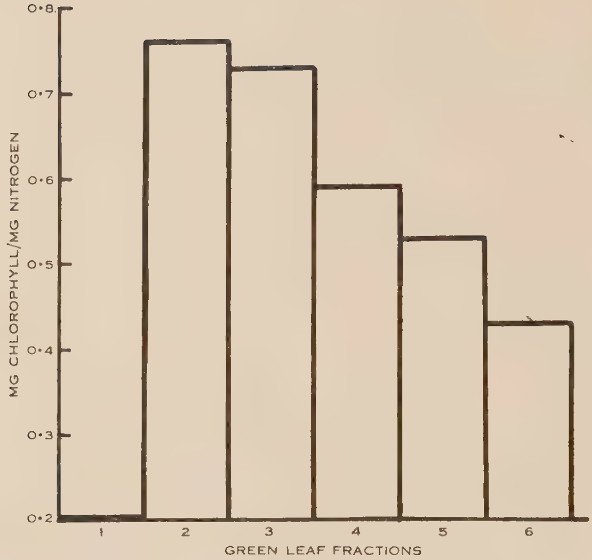

Fig. 3.—The distribution of chlorophyll in particulate fractions from green leaves.

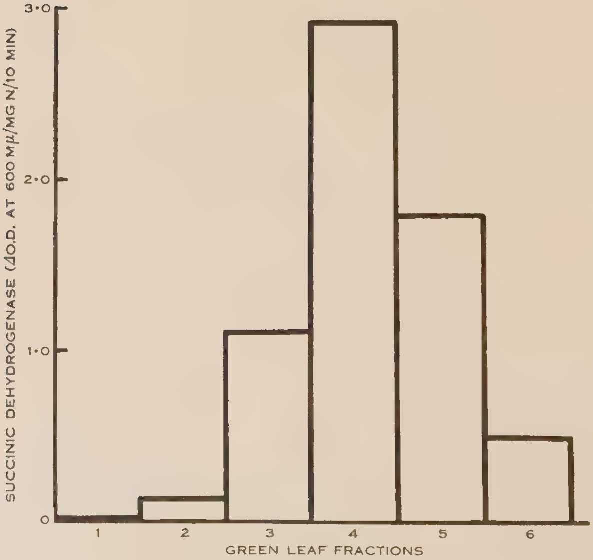

Fig. 4.—The distribution of succinic dehydrogenase in green leaf fractions. Reaction mixture contained enzyme (0·1 ml), potassium phosphate at pH 6·8 (0·05M), egg albumin (1 mg), 2,6-dichlorophenolindophenol (0·0001M), and succinate (0·02M). Final volume 6·0 ml.

(c) *Distribution of Starch*

Eighty-nine per cent. of the starch was sedimented in fraction 1 (Table 2). This would indicate that a high proportion of the chloroplasts are partially disrupted during the extraction procedure. Microscopic examination of the fractions also showed that the majority of starch grains were in fraction 1.

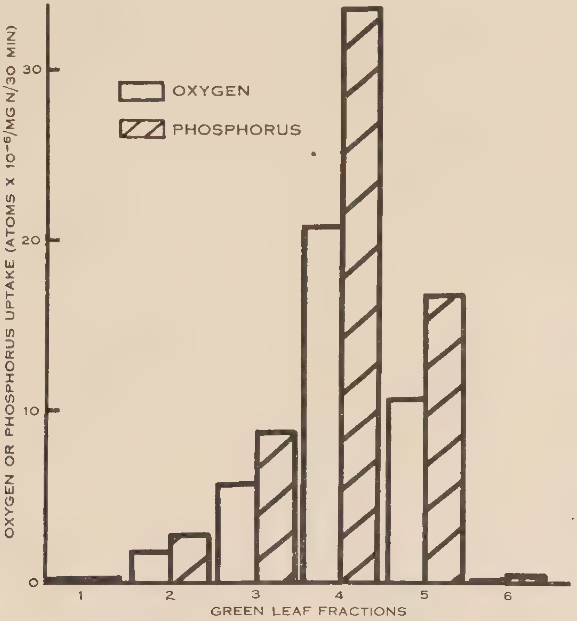

Fig. 5.—The distribution of oxidative and phosphorylative capacities of particulate fractions from green leaves. Reaction mixture contained enzyme, sucrose (0·35M), potassium phosphate at pH 7·3 (0·03M), $MgSO_4$, (0·001M), adenylate (0·0005M), yeast coenzyme concentrate (1 mg), glucose (0·002M), hexokinase (0·2 mg), and succinate (0·02M). Final volume 2·0 ml.

(d) *Distribution of Chlorophyll and Photolytic Activity*

The distribution of chlorophyll is shown in Figure 3. There was a gradual decrease in the chlorophyll: total nitrogen ratio from fractions 2 to 5. Fractions 2 and 3 contained the highest amount of chloroplastic material. Jagendorf and Wildman (1954) found a chlorophyll: total nitrogen ratio of 1·0 for purified tobacco leaf chloroplasts. No value is available for pea leaf chloroplasts, but assuming it is approximately 1·0, this would indicate that approximately 25 per cent. of fractions 2 and 3 were non-chloroplastic in origin, while in fraction 6 over 50 per cent. was of non-chloroplastic origin.

The photolytic capacity of the particles in the presence of ferricyanide did not follow the chlorophyll distribution precisely, the ratio of mg chlorophyll to photolytic activity (μl O_2 in 25 min $\times 10^{-2}$) being 1·52, 1·17, 0·98, 0·83, and 0·72 for fractions

2, 3, 4, 5, and 6 respectively. This result might be expected if it is assumed photolysis is a surface phenomenon (Milner, Koenig, and Lawrence 1950), since the fragments in fraction 6 would contain more of the grana components.

(*e*) *Mitochondrial Activity*

The distribution of succinic dehydrogenase activity is shown in Figure 4. The rates of oxygen uptake and phosphorus esterification in the presence of succinate associated with fractions 1–6 are summarized in Figure 5. The rate of oxidation of α-ketoglutarate was similar to that of succinate oxidation. Results obtained with different preparations were similar in each case and almost identical to those already found for etiolated leaves (Smillie 1956*b*). The rate of phosphorylation closely followed the oxidative rate, the ratio of phosphorus esterified to oxygen consumed (P:O) for fractions 2, 3, 4, and 5 being 1·52, 1·53, 1·60, and 1·52 respectively.

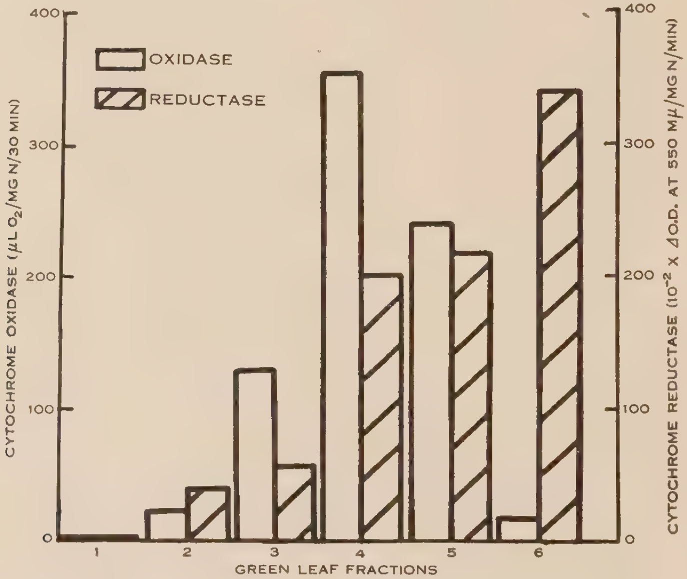

Fig. 6.—The distribution of cytochrome oxidase and DPN-linked cytochrome reductase in particulate fractions from green leaves. For cytochrome oxidase determinations the reaction mixture contained enzyme, potassium phosphate at pH 7·3 (0·01M), cytochrome *c* (10^{-5}M), and *p*-phenylenediamine (0·02M). Final volume 2·0 ml. For cytochrome reductase determinations the reaction mixture contained enzyme, potassium phosphate at pH 7·3 (0·01M), oxidized cytochrome *c* ($5{\cdot}2\times10^{-9}$M), yeast coenzyme concentrate (1 mg), alcohol dehydrogenase (0·2 mg), KCN (0·002M), and ethanol (0·1M). Final volume 2·0 ml.

(*f*) *Distribution of Cytochrome Oxidase and Cytochrome Reductase*

Cytochrome oxidase has a similar distribution to the oxidative activities already described (Fig. 6). Cytochrome reductase was located in particles whose average size were smaller than the mitochondria (Fig. 6).

(*g*) *Microscopic Examination of Green Leaf Fractions*

The different fractions were examined by phase-contrast microscopy. Fraction 1 contained many starch grains and whole nuclei. Fraction 2 also contained whole nuclei and a smaller number of starch grains, but these were absent from the subsequent fractions. Whole chloroplasts were confined to fractions 2 and 3. Although particles 2 μ in diameter or less were observed in all fractions except fraction 1, there was a decrease in the average size of these particles through fractions 2 to 6. In Table 3 are shown the distribution of particles 0·5–2 μ in diameter and whole chloroplasts or chloroplast fragments. Many of the particles less than 2 μ in diameter were irregular in shape, suggesting that they were also chloroplast fragments. In addition, fractions 2, 3, and 4 contained larger particles, which were obviously fragmented chloroplasts. The particles in fraction 6 were at the limit of resolution of the microscope, and too small for counting.

TABLE 3

DISTRIBUTION OF WHOLE CHLOROPLASTS AND SMALLER PARTICLES IN GREEN LEAF FRACTIONS

Fraction	Particles ($\times 10^9$/mg N)		
	Whole Chloroplasts	Chloroplast Fragments (>2 μ)	Particles (0·5–2 μ)
1	0	0	0
2	2·1	1·3	3·0
3	3·3	4·9	25·4
4	Trace	3·4	57·0
5	0	Trace	69·0
6	0	0	Not counted

IV. DISCUSSION

The procedure used in preparing the leaf homogenate resulted in the rupture of many whole chloroplasts. This is in contrast with results obtained with etiolated leaves, where all the plastids were isolated in one fraction. The presence of starch grains in the chloroplasts probably contributed to their ease of rupture during grinding. Although chlorophyll-containing material was present in all fractions except fraction 1, it is obvious from the distribution patterns obtained, that the mitochondrial activities were due to particles which were quite distinct from chloroplasts. The distribution of chlorophyll and the photolytic activity varied in different preparations, but the distribution patterns of the other enzymic activities were reproducible and were strikingly similar to results obtained for etiolated leaves (Smillie 1956*b*).

Recently Ohmura (1955) has obtained a particulate preparation from spinach leaves which carried out oxidative phosphorylation in the presence of citrate. This activity he attributed to chloroplast fragments. In view of the results described above, it is more probable that the activity was due entirely to enzymes located on mitochondrial particles which were also present in the leaf preparation.

In both etiolated and green leaves there exist at least four distinct types of particles—nuclei, plastids, mitochondria, and cytochrome reductase-containing particles lacking oxidative activity. Of these particles, both the plastids and the cytochrome reductase-containing particles possess higher amounts of RNA than the mitochondria. It is to be noted that Jagendorf and Wildman (1954) found very little nucleic acid in chloroplasts from tobacco leaves. However, the experiments described above were performed with rapidly growing leaves, whereas those of Jagendorf and Wildman were carried out with mature leaves in which the growth and division of chloroplasts was at a minimum (Strugger 1950). Since RNA has been associated with protein synthesis (Brachet 1950), the RNA level in chloroplasts might be expected to fall as the leaf matures. Similarly the number of non-chloroplastic cytoplasmic particles which are concerned with energy production and cell synthesis may also decrease as the leaf matures.

V. Conclusion

In the growing pea plant, the cellular make-up and the enzyme distribution within the leaf cell is fundamentally similar whether the plant is grown in the presence or absence of light. The leaves contain in addition to the plastids, other cytoplasmic bodies, namely, mitochondria and particles corresponding in size to animal microsomes. The mitochondria and possibly the microsomes function in respiratory processes which lead to the production of energy for cellular synthesis, and which are quite distinct from photosynthetic mechanisms located in the chloroplast. Both the mitochondria and microsomes are present in large numbers in the rapidly growing leaf. These results, which were obtained with young leaves, may not be typical of mature leaves.

VI. Acknowledgments

The author is indebted to the Commonwealth Scientific and Industrial Research Organization for financial assistance, and to the Research Committee, University of Sydney, for purchase of chemicals. The author also wishes to thank Dr. Adele Millerd for her helpful comments.

VII. References

Arnon, D. I. (1949).—*Plant Physiol.* **24**: 3.

Arnon, D. I., Allen, M. B., and Whatley, F. R. (1954).—*Nature* **174**: 394.

Arnon, D. I., and Whatley, F. R. (1949).—*Arch. Biochem.* **23**: 141.

Arnon, D. I., and Whatley, F. R. (1954).—*Physiol. Plant.* **7**: 602.

Brachet, G. (1950).—*Ann. New York Acad. Sci.* **50**: 861.

Drury, H. F. (1948).—*Arch. Biochem.* **19**: 455.

Jagendorf, A., and Wildman, S. G. (1954).—*Plant Physiol.* **29**: 270.

McClendon, J. H. (1953).—*Amer. J. Bot.* **40**: 260.

Milner, H. W., Koenig, M. L. G., and Lawrence, N. S. (1950).—*Arch. Biochem.* **28**: 185.

Ohmura, T. (1955).—*Arch. Biochem. Biophys.* **57**: 187.

Smillie, R. M. (1956*a*).—*Aust. J. Biol. Sci.* **9**: 81.

Smillie, R. M. (1956*b*).—*Aust. J. Biol. Sci.* **9**: 339.

Strugger, S. (1950).—*Naturwissenschaften* **37**: 166.

NUCLEIC ACID CONTENT OF PETKUS RYE EMBRYOS IN RELATION TO VERNALIZATION AND DEVERNALIZATION

By L. R. Finch* and D. J. Carr†

[*Manuscript received December 29, 1955*]

Summary

Petkus winter rye has been subjected to vernalization treatments using the technique of restricted moisture content in plastic boxes lined with filter paper. Grain so treated has also been subjected to the devernalizing action of temperatures higher than those at which vernalization proceeds.

Determinations have been made of dry weight, nitrogen, acid-soluble phosphorus, ribonucleic acid-phosphorus and deoxyribonucleic acid-phosphorus in embryos excised from the grain. These do not give evidence of changes, related to vernalization, in any of the contents of the phosphorus fractions, including ribonucleic acid.

I. Introduction

The self-perpetuating nature of the flowering stimulus, both in vernalization and in photoperiodism, has been recognized (Lona 1947; Purvis 1948; Lang 1952) and this has led to the postulation that substances produced auto-catalytically are involved in floral initiation. The inductive stimuli of cold or daylength would thus be expected to bring about the initial production or accumulation of such substances. As the sum of the existing evidence indicates that the flowering stimulus may be transferred with certainty only through a living tissue junction, it seems that the stimulatory material may consist of complex substances. The analogy between the inductive processes involved in flowering and those involved in the formation of tumours by crown gall bacteria suggests similarities in the nature of the inductive principles. This suggestion is heightened by the auto-catalytic nature of the tumour-inducing principle and its transmissibility by grafting. Klein (1953) believes that the tumour-inducing principle may be a "special polymer of DNA". He reports a 100 per cent. increase in deoxyribonucleic acid (DNA) content of tomato stems during the inductive phase of tumour genesis. Changes in nucleic acid might therefore be expected to occur during the process of induction of flowering. Ehrenberg (1954) has reported a change in the diurnal rhythm of phosphatase activity in *Kalanchoë Blossfeldiana* under photo-inductive daylengths, and this could be evidence of the initiation of changes in phosphate metabolism, possibly involving the phosphate of nucleic acid.

There are at least two ways in which changes in nucleic acid metabolism might be related to the induction of flower formation. One possibility is that such changes could lead to the formation of a nucleic acid material specific for flowering. Alternatively, during induction, a reserve of non-specific nucleic acid might accumulate, and this might be utilized in the subsequent elaboration of specific substances involved in flower initiation. The recognition of a specific nucleic acid material would be

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difficult by existing chemical techniques, but any accumulation, such as that found by Klein in tumour induction, might be readily detected.

Since the conditions of low temperature under which induction of flowering (vernalization) occurs in Petkus winter rye are well defined (Gregory and Purvis 1938*a*, 1938*b*), studies of the nucleic acid content of embryos of this plant during vernalization were considered to be the most profitable line of initial investigation. Before the experiments were started, results published by Konarev (1954) indicated that cold treatment produced marked increases in the ribonucleic acid (RNA) content of embryos of Saratov No. 1 winter rye and Lutescens 329 winter wheat, both of which are susceptible to vernalization, whereas no such change occurred in the embryos of the spring wheat Lutescens 62, which does not respond to vernalization. The results of our investigation do not show similar changes of contents of nucleic acid in embryos of Petkus winter rye during vernalization with restricted moisture content.

II. METHODS

(*a*) *Treatment of Grain*

Petkus winter rye* (100 g) was rinsed twice with warm tap water and three times with distilled water, and then shaken with 100 ml of a suspension of "Ceresan-M" seed sterilant, 1/10,000 in water. Ragai and Loomis (1954) have found the use of 1/10,000 "Ceresan-M" gives complete sterilization of maize grain without any effect on respiration and with only a 5 per cent. reduction in germination. The grain was spread out in an 8×12 in. porcelain dish into which the suspension of "Ceresan-M" was poured. Excess liquid was decanted so that the grain was not covered. The grain was incubated at 20°C for 6 hr, and then transferred to "vernalization boxes" and incubated for a further 24 hr at 20°C.

The vernalization boxes were 5×4 in. polyvinyl acetate containers, $2\frac{1}{2}$ in. deep, lined with Whatman No. 1 filter paper, and covered with a layer of filter paper and polythene sheet sealed with "Cellulose" adhesive tape. Gas exchange was allowed by six 2-mm holes drilled around the sides of each box. A window was cut in the filter paper which lined the sides of each box to allow inspection of the grain. 20 g of moist grain (about 450 individual grains) was spread evenly on the bottom of each box and the filter paper moistened with water.

The amount of water required to wet the filter paper evenly was determined as 0·57 ml per g dry weight of filter paper and the paper was moistened at this ratio in the first experiment. As this treatment gave rise to grain with a moisture content below 50 per cent. of the dry weight, a ratio of 1·0 ml of water per g of filter paper was used in the second experiment. This proved satisfactory in giving grain of a moisture content of just over 50 per cent. of the dry weight.

At the time of transfer to the boxes the moist grain was sampled for dry weight determination. At the completion of the 24-hr incubation period the amount of water needed to bring the moisture content of the grain to 50 per cent. of the dry weight (i.e. dry weight = 66·7 per cent. moist weight) was added to the boxes. The boxes and their contents were then placed in a cold room (temperature 0–4°C). They were weighed weekly and losses in weight made up by the addition of water.

* Kindly supplied by the National Institute of Agricultural Botany, Cambridge.

At the completion of the period of vernalization a sample of 3–4 g of grain was taken for dry weight determination. The remainder of the grain was divided between three dishes of the type used by Purvis and Gregory (1952). These dishes were then subjected to temperatures of 0–4, 20, and 35°C, respectively, for 66–68 hr, after which the grain was analysed.

(*b*) *Sampling*

Embryos were dissected from the grain and placed in tared glass homogenizer tubes for the extraction, and in tared micro-Kjeldahl flasks for the determination of dry weight and of nitrogen content. The homogenizer tubes were suitable for centrifuging at the various stages of the extraction. About 20 embryos were taken for the extraction procedure and 10–15 for the dry weight and nitrogen determination. Duplicate samples were taken both for extraction and for determination of dry weight and of nitrogen content. Embryos were selected to be of as uniform size as possible, over-large and over-small embryos being discarded.

(*c*) *Extraction*

Extraction and estimation of acid-soluble phosphorus, RNA-phosphorus, and DNA-phosphorus was carried out using the method of Ogur and Rosen (1950), as modified by Martin and Morton (private communication, 1954). The method is described here for convenience. Immediately after weighing, the samples for extraction were homogenized with 3 ml of methanol, $1\frac{1}{2}$ ml being placed in the tube initially and the further $1\frac{1}{2}$ ml being used to wash down the pestle after homogenizing. The tubes were centrifuged and the methanol extract discarded. Homogenizing as above was repeated and the methanol extraction was allowed to continue for 30 min, after which 3 ml of chloroform was added and a further 30 min extraction was allowed. The residue was again homogenized with 3 ml of methanol, then 3 ml of chloroform was added to the suspension immediately, and the extraction continued for 30 min. The extract was again discarded while the residue was washed twice with 6 ml of ether and then dried in a desiccator evacuated continuously overnight.

The residue was homogenized, as with methanol, with 3 ml of ice-cold water and then 2 ml of ice-cold 0·5N perchloric acid was added and the suspension centrifuged immediately, using the centrifuge in a cold room at 0–4°C. The extract was retained and the procedure repeated twice, the combined extracts being made up to 15 ml and used for the determination of acid-soluble phosphorus.

The residue was shaken with 5 ml N perchloric acid at 0°C for 24 hr, the suspension was centrifuged and the extract was retained. This procedure was repeated for a 6-hr extraction and for an 18-hr extraction. The combined extracts were made up to 15 ml, and used for the determination of RNA-phosphorus and ultraviolet absorption.

The residue was suspended in 5 ml 10 per cent. sodium chloride in 0·1M acetate buffer, pH 4, and heated in a boiling water-bath for 20 min. The suspension was centrifuged and the extract retained. There followed two washes each of 2 3 min in a boiling water-bath with 5 ml of the buffered saline. The combined extracts were made up to 15 ml and used for the determination of DNA-phosphorus.

TABLE 1

ANALYSES OF EMBRYOS FROM GRAIN TREATED AT MOISTURE CONTENTS RESTRICTED TO APPROXIMATELY 43% OF THE DRY WEIGHT

Length of Initial Cold Treatment (weeks)	Whole Grain Dry Wt./ Moist Wt. (%)	66-Hr Incubation Temp. (°C)	N Determination		P Determination	N/ Dry Wt. (%)	Acid-soluble P/ Dry Wt. (%)	RNA-P/ Dry Wt. (%)	DNA-P/ Dry Wt. (%)	Acid-soluble P/ Embryo (μg)	RNA-P/ Embryo (μg)	DNA-P/ Embryo (μg)	Acid-soluble P/N (%)	RNA-P/N (%)	DNA-P/N (%)
			Dry Wt./ Moist Wt. (%)	Dry Wt. (mg)	Dry Wt.* (mg)										
0	—	0	52·7 47·3	0·92 0·86	0·94 0·91	9·0 8·6	0·64 0·65	0·30 0·30	0·13 0·15	6·0 5·9	2·8 2·7	1·3 1·4	7·4	3·4	1·6
		20	41·0 43·8	1·00 0·90	1·07 1·13	7·9 8·1	0·33 0·36	0·28 0·30	0·12 0·12	3·5 4·1	3·0 3·4	1·3 1·4	4·3	3·6	1·5
		35	53·7 53·8	1·23 1·10	0·97 1·07	7·0 7·7	0·51 —	0·27 —	0·12 —	5·0 —	2·6 —	1·2 —	6·9	3·7	1·6
3	69·0	0	67·4 67·7	0·94 0·96	0·91 0·91	9·4 —	0·56 0·57	0·26 0·27	0·13 0·16	5·1 5·2	2·4 2·5	1·2 1·5	5·9	2·8	1·5
		20	37·4 39·6	1·00 0·92	1·02 1·03	8·6 8·8	0·27 0·30	0·26 0·27	0·14 0·15	2·6 3·1	2·6 2·8	1·4 1·5	3·2	3·0	1·6
		35	60·0 57·7	0·77 0·80	0·88 1·08	7·8 8·0	0·46 0·43	0·27 0·25	0·15 0·12	4·1 4·6	2·4 2·7	1·3 1·3	5·6	3·3	1·7
6	70·0	0	58·0 59·5	0·63 0·63	0·66 0·62	9·1 8·7	0·86 0·83	0·36 0·38	0·19 0·21	5·7 5·2	2·4 2·4	1·3 1·3	9·4	4·1	2·2
		20	40·0 40·5	0·60 0·64	0·62 0·66	8·5 8·2	0·63 0·62	0·38 0·38	0·17 0·18	3·9 4·1	2·4 2·5	1·1 1·2	7·4	4·5	2·1
		35	53·3 56·7	0·63 0·71	0·66 0·65	7·7 7·8	0·60 0·57	0·35 0·34	0·19 0·18	4·0 3·7	2·3 2·2	1·3 1·2	7·5	4·4	2·4
8	69·5	0	53·0 51·8	0·88 0·63	0·74 0·62	9·2 9·3	0·43 0·52	0·35 0·40	0·16 0·18	3·2 3·2	2·6 2·5	1·2 1·1	5·1	4·0	1·8
		20	62·6 60·0	0·71 0·67	0·70 0·70	8·8 8·5	0·48 0·41	0·33 0·34	0·21 0·20	3·4 2·9	2·3 2·4	1·5 1·4	5·1	3·8	2·3
		35	63·5	0·70	0·71	7·8	0·61	0·32	0·21	4·3	2·3	1·5	7·8	4·1	2·7

* These values were calculated by multiplying the average moist weight of the embryos by the average of the ratio (dry weight/moist weight) for the embryos used for the corresponding nitrogen determinations.

It is doubtful whether there is any method available (including the one used here) for the complete quantitative separation and estimation of RNA and DNA. However, the agreement obtained for duplicates indicates that the present method gives reproducible fractions. For the RNA fractions, the ratio of optical density at 260 mμ to μg of phosphorus (0·30–0·35 in the first experiment and 0·34–0·36 in the second experiment) indicates that this fraction is of fairly constant composition.

Paper chromatography of perchloric acid hydrolysates by the method of Wyatt (1951) showed the RNA extract to contain uracil, but no thymine. Chromatograms were not conclusive as to the presence, or absence, of uracil in hydrolysates of the DNA extract.

Thus, although it seems likely that the RNA extract was free of DNA contamination, the purity of the DNA extract cannot be assured.

(*d*) *Ultraviolet Absorption*

Ultraviolet absorption was determined over the range 240–290 mμ in a Beckman DU spectrophotometer using cuvettes of 1 cm path length. Absorption was always a maximum at 260 mμ for the RNA extract, and the optical density at this wavelength was taken as one measure of the RNA concentration.

(*e*) *Phosphorus Estimation*

An aliquot of extract containing 2–6 μg of phosphorus was taken and mixed with a quantity of 10N sulphuric acid in a 1-in. diameter "Quickfit" stoppered tube. 0·5 ml of 10N sulphuric acid was used per ml of acid-soluble extract and of RNA extract, but an additional 0·2 ml was added to each ml of 10 per cent. salt extract of DNA taken. One drop of 70 per cent. perchloric acid was also added to the mixture for DNA-phosphorus estimation. The mixtures were then evaporated to fumes over a micro-burner and placed on a heating rack for 15 min, after which time the line of condensation was 1–2 in. up the tube. Standards of 3·0, 6·0, and 9·0 μg of phosphorus as phosphate were also carried through the digestion. The digests were cooled, 5 ml of distilled water was added to them, and they were incubated for 10 min in a boiling water-bath to hydrolyse pyrophosphate. On cooling, phosphate-phosphorus was estimated by the method of Weil-Malherbe and Green (1951).

(*f*) *Dry Weight Determination*

After dissection and weighing, the samples for the determination of dry weight were put in an oven at 100°C for 8 hr, and re-weighed after cooling over pellets of sodium hydroxide in a desiccator. They were then used for the estimation of nitrogen.

(*g*) *Nitrogen Estimation*

Digestion was performed with 0·5 ml of a reagent containing selenium dioxide (0·33 per cent. w/v) and cupric sulphate pentahydrate (0·67 per cent. w/v) in 48 per cent. v/v sulphuric acid. Dissolution of the embryos was speeded by the addition of drops of 100 volume hydrogen peroxide to the cooled digests. Digestion was continued for 4 hr after evaporation of water. The digest was made up to 25 ml with distilled water, and ammonium-nitrogen was determined by Nesslerization (King

TABLE 2

ANALYSES OF EMBRYOS FROM GRAIN TREATED AT MOISTURE CONTENTS RESTRICTED TO APPROXIMATELY 50% OF THE DRY WEIGHT

Length of Initial Cold Treatment (weeks)	Whole Grain Dry Wt./ Moist Wt. (%)	66-Hr Incubation Temp. (°C)	N Determination		P Determination	N/ Dry Wt. (%)	Acid-soluble P/ Dry Wt. (%)	RNA-P/ Dry Wt. (%)	DNA-P/ Dry Wt. (%)	Acid-soluble P/ Embryo (μg)	RNA-P/ Embryo (μg)	DNA-P/ Embryo (μg)	Acid-soluble P/N (%)	RNA-P/N (%)	DNA-P/N (%)
			Dry Wt./ Moist Wt. (%)	Dry Wt. (mg)	Dry Wt.* (mg)										
4	65·6	0	46·0 45·0	1·00 1·24	1·12 1·32	8·0 7·8	0·27 0·27	0·30 0·29	0·12 0·13	3·0 3·6	3·4 3·8	1·3 1·7	3·4	3·7	1·5
		20	50·5 43·7	1·36 1·21	1·17 1·25	7·0 —	0·29 0·32	0·26 0·27	0·13 0·12	3·4 4·0	3·0 3·4	1·5 1·5	4·3	3·8	1·8
		35	56·0 54·0	1·64 1·57	1·34 1·17	6·2 6·6	0·28 0·28	0·26 0·27	0·11 0·11	3·8 3·3	3·7 3·2	1·5 1·3	4·4	4·1	1·7
6	65·0	0	42·4 41·4	1·00 1·09	1·06 1·01	8·5 8·3	0·28 0·28	0·29 0·28	0·12 0·13	3·0 2·8	3·1 2·8	1·3 1·3	3·3	3·3	1·4
		20	43·8 42·0	1·42 1·44	1·70 2·01	7·3 7·0	0·23 0·24	0·23 0·22	0·11 0·11	3·9 4·8	3·9 4·4	1·9 2·2	3·3	3·1	1·5
		35	50·6 50·6	1·88 1·57	1·81 1·61	6·2 6·8	0·22 0·26	0·24 0·26	0·11 0·10	4·0 4·2	4·3 4·2	2·0 1·6	3·7	3·8	1·6
8	66·5	0	52·8 51·4	1·92 1·42	1·45 1·38	8·6 8·3	0·28 0·29	0·29 0·27	0·12 0·11	4·0 4·0	4·2 3·7	1·7 1·5	3·3	3·3	1·3
		20	41·0 43·6	2·00 2·04	2·06 2·12	7·4 7·0	0·25 0·24	0·22 0·23	0·11 0·11	5·1 5·0	4·5 4·8	2·3 2·3	3·4	3·1	1·5
		35	56·7 58·2	1·96 1·98	1·94 2·06	6·8 6·6	0·23 0·24	0·22 0·22	0·10 0·10	4·5 4·9	4·3 4·5	1·9 2·1	3·5	3·3	1·5

* These values were calculated by multiplying the average moist weight of the embryos by the average of the ratio (dry weight/moist weight) for the embryos used for the corresponding nitrogen determinations.

1951). Blanks were carried through the digestion procedure, while standards of 6, 12, 24, and 36 μg of ammonium-nitrogen were carried through the Nesslerization.

III. Results and Discussion

Gregory and Purvis (1938*a*, 1938*b*) have found that the degree of vernalization of Petkus winter rye, at a temperature of 0–4°C, is progressively increased for increasing duration of cold treatment up to 8 weeks. After 8 weeks of cold treatment the vernalization process is almost complete, and there is little decrease of the degree of vernalization with subsequent treatments at temperatures from 15 to 40°C. However, the decrease of the degree of vernalization (devernalization) is considerable for grain which is incompletely vernalized as a result of having less than 8 weeks of cold treatment. The processes of both vernalization and devernalization can be carried out on grain with a moisture content restricted to 50 per cent. of the dry weight (i.e. dry weight = 66·7 per cent. of moist weight), to prevent excessive growth of the embryos (Purvis and Gregory 1952). Embryos excised from the grain and grown on sugar can be vernalized, so that the locus of the process is in the embryo (Gregory and Purvis 1938*a*).

On the basis of these findings, two experiments were carried out on Petkus winter rye to test whether there were changes in the contents of acid-soluble phosphorus, RNA-phosphorus, or DNA-phosphorus in the embryos of grain treated under the conditions causing vernalization and devernalization. The technique of restricted water content was used to limit growth of the embryos in order that they would remain of roughly comparable size in all treatments.

In the first experiment (Table 1) cold treatments were applied for 3, 6, and 8 weeks with incubations for 66–68 hr at 0, 20, and 35°C at the conclusion of each cold treatment. The moisture content of the grain after cold treatment was of the order of only 43 per cent. of the dry weight (dry weight = 70 per cent. of moist weight). Grain in the control series received the initial soaking and incubation at 20°C plus the further incubation for 66 hr at the three post-vernalization temperatures. There was a rise in RNA-phosphorus/unit dry weight from the control (no cold treatment) to the 6 and 8 weeks cold treatment, but this rise is possibly related to the concurrent fall in embryo dry weight which may have resulted from over-restriction of the water content of the cold-treated grain. This over-restriction of the water content makes the results of the experiment of uncertain validity with respect to vernalization. They have been included here since this trend of RNA-phosphorus/unit dry weight is in line with the increase found by Konarev (1954) during vernalization. However, the trend is reversed when the results are expressed as RNA-phosphorus per embryo, and was absent in the second experiment in which the moisture content was approximately 50 per cent. The fall in RNA-phosphorus/unit dry weight, with devernalizing treatments after 6 and 8 weeks cold treatment, is too slight to be of significance, and is reversed, or reduced, when expressed on a basis of unit nitrogen, or per embryo.

In the second experiment (Table 2) cold treatments were applied for 4, 6, and 8 weeks with incubations for 66–68 hr at 0, 20, and 35°C at the conclusion of each cold treatment. The moisture content of the whole grain after cold treatment was just

TABLE 3

ANALYSES OF EMBRYOS FROM GRAIN INCUBATED AT MOISTURE CONTENTS RESTRICTED TO APPROXIMATELY 50% OF THE DRY WEIGHT

Period of Incubation at 20°C (hr)	N Determination		P Determination	N/Dry Wt. (%)	Acid-soluble P/Dry Wt. (%)	RNA-P/ Dry Wt. (%)	DNA-P/ Dry Wt. (%)	Acid-soluble P/Embryo (μg)	RNA-P/ Embryo (μg)	DNA-P/ Embryo (μg)	Acid-soluble P/N (%)	RNA-P/N (%)	DNA-P/N (%)
	Dry Wt./ Moist Wt. (%)	Dry Wt. (mg)	Dry Wt.* (mg)										
24	38·0	1·03	1·16	7·0	0·26	0·29	0·13	3·0	3·4	1·5	3·8	4·2	2·0
	36·0	1·13	1·08	6·6	0·26	0·28	0·14	2·8	3·0	1·5			
48	40·3	1·45	1·44	6·3	0·25	0·26	0·13	3·6	3·7	1·9	4·1	4·4	2·1
	39·8	1·41	1·39	6·0	0·25	0·27	0·13	3·5	3·8	1·8			
72	41·2	1·87	1·83	6·1	0·24	0·24	0·11	4·4	4·4	2·0	3·9	4·0	1·9
	41·9	1·80	1·86	5·8	0·23	0·24	0·12	4·3	4·5	2·2			

* These values were calculated by multiplying the average moist weight of the embryos by the average of the ratio (dry weight/moist weight) for the embryos used for the corresponding nitrogen determinations.

over 50 per cent. of the dry weight (dry weight = 66·7 per cent. of moist weight). The grain in the control series (Table 3) was soaked and incubated for 24, 48, and 72 hr at 20°C to produce embryos of a range of size, measured as dry weight, but not subjected to conditions affecting vernalization. For this experiment, there is no change in RNA-phosphorus/unit dry weight during the period of vernalization. There is an increase in RNA-phosphorus/embryo with cold treatment (Table 2); but the similar increase in RNA-phosphorus/embryo with increasing embryo size measured as dry weight in the control series (Table 3) indicates that this is a function of embryo size rather than degree of vernalization. Similarly, the fall in RNA-phosphorus/unit dry weight under the devernalizing high temperature treatments appears to be related to embryo size rather than to devernalization.

Similarly, no changes related to vernalization can be seen in the acid-soluble or DNA-phosphorus fractions estimated in either experiment. Nitrogen content falls with high temperature treatment so that all the phosphorus fractions appear to increase with high temperature treatment when expressed on a basis of unit nitrogen (Table 2).

Thus, no increase in RNA-phosphorus of the magnitude of that found by Konarev (1954) for winter rye (Saratov No. 1) or winter wheat (Lutescens 329) was found in these experiments on Petkus winter rye. The difference between our results and those of Konarev may be related to our use of the technique of restricted moisture content. This technique prevents excessive growth and changes in dry weight of the embryos undergoing cold treatment while permitting vernalization to proceed unchecked. Since there is no apparent trend in either DNA-phosphorus or RNA-phosphorus with the progress of vernalization, it is not surprising that the devernalization treatments have little effect on these fractions other than that which is shown by non-vernalized grain subjected to the same temperature treatments, and which appears to be due to growth and increase in embryo dry weight.

Though these analyses do not indicate the likelihood of *gross* change in nucleic acid fractions during vernalization of Petkus winter rye, they do not exclude the possibility of the elaboration of a *specific* nucleic acid-containing substance in the process.

IV. REFERENCES

EHRENBERG, M. (1954).—*Planta* **43**: 528.

GREGORY, F. G., and PURVIS, O. N. (1938*a*).—*Ann. Bot., Lond.* (N.S.) **2**: 237.

GREGORY, F. G., and PURVIS, O. N. (1938*b*).—*Ann. Bot., Lond.* (N.S.) **2**: 753.

KING, E. J. (1951).—"Microanalysis in Medical Biochemistry." 2nd Ed. p. 9. (J. & A. Churchill Ltd.: London.)

KLEIN, R. M. (1953).—*Amer. J. Bot.* **40**: 597.

KONAREV, V. G. (1954).—*Biochimia* **19**: 131.

LANG, A. (1952).—*Annu. Rev. Pl. Physiol.* **3**: 265.

LONA, F. (1947).—*Lavori di Botanica, Padova* **1947**: 277.

OGUR, M., and ROSEN, G. (1950).—*Arch. Biochem.* **25**: 262.

PURVIS, O. N. (1948).—*Ann. Bot., Lond.* (N.S.) **12**: 183.

PURVIS, O. N., and GREGORY, F. G. (1952).—*Ann. Bot., Lond.* (N.S.) **16**: 1.

RAGAI, H., and LOOMIS, W. E. (1954).—*Plant Physiol.* **29**: 49.

WEIL-MALHERBE, H., and GREEN, R. H. (1951).—*Biochem. J.* **49**: 286.

WYATT, G. R. (1951).—*Biochem. J.* **48**: 584.

THE THERMOCHEMISTRY OF BIOLOGICAL NITROGEN FIXATION

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Summary

Using standard thermodynamic data it is shown that the oxidation of glucose by nitrogen to give carbon dioxide and ammonia is an overall reaction that involves a substantial evolution of heat and decrease in free energy. This is not in accordance with literature statements that biological nitrogen fixation is endothermic. In the concluding section a Frost diagram is used to survey the thermodynamic data of oxidation reactions related to nitrogen fixation.

I. Introduction

The literature on biological nitrogen fixation contains a number of references to the alleged energetically unfavourable nature of the fixation process, with the suggestion that it requires assistance from the energy supplied by other processes, such as respiration. Thus a recent review by Fogg (1955) states that "fixation must be an endothermic reaction," and Pethica, Roberts, and Winter (1954) use the words "it is generally conceded that the fixation process, taken as a whole, is endothermic." In an earlier review on energetic coupling, Kalckar (1941) states that the reduction of the triple bond in nitrogen is an endergonic reaction. Of these authors only Kalckar quotes a reference, which is without a page number, to Lewis (1923), a work in which the present author has been unable to find a relevant statement except one relating to the well-known great stability of the N≡N bond.

On the other hand, nearly thirty years ago Burk (1927) presented thermodynamic evidence against the (then) current conception that energy was *necessarily* required for the fixation of nitrogen. However, Burk's particular examples of fixation that could occur with the liberation of energy or free energy were confined to reactions that involved the participation of oxygen gas, or hydrogen gas, or both, "or other substances, especially gases, whose standard free energies are close to zero." In his concluding remarks he raised the question of the carbohydrate requirement of nitrogen-fixing organisms and suggested some unobserved function for carbohydrate, together with the possibility that fixation can make energy available for use in general metabolism.

More recently Wilson and Burris (1947), in a general review of mechanisms of nitrogen fixation, have discussed thermodynamic aspects of fixation by *Azotobacter* in terms of definite evidence that ammonia is the key intermediate and that glucose can be regarded as a typical nutrient medium. They considered the overall reaction in which the reduction of nitrogen to ammonia is brought about by the "metabolic hydrogen" of glucose (a reaction which chemically could equally well be described as the oxidation of glucose by nitrogen). Taking into account reasonable concentrations of nutrient, atmospheric nitrogen, evolved carbon dioxide, and the requirements

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of a pH = 7, their overall equation is the following:

$$C_6H_{12}O_6(0{\cdot}05M)+4N_2(0{\cdot}8\text{ atm})+18{\cdot}67H_2O(\text{liq.}) = 8NH_4^+(10^{-4}M)+8OH^-(10^{-7}M)$$
$$+4{\cdot}67HCO_3^-(0{\cdot}0012M)+4{\cdot}67H^+(10^{-7}M)+1.33CO_2(0{\cdot}01\text{ atm}) \quad \ldots.(1)$$

Of this reaction Wilson and Burris stated "*Although* the *standard* free energy of this reaction (ΔG^0)* is +5·6 kg cal per 0·5N$_2$, the organism *actually obtains* energy by carrying out the reaction under the specified conditions, ΔG equalling −17·5 kg cal per 0·5 mole N$_2$ fixed" (italics are the present author's). While it is clear that Wilson and Burris envisaged the oxidation of glucose by nitrogen in terms of the overall equation (**1**) as a reaction which can proceed without assistance, given a suitable mechanism, their use of the words italicized by the author may suggest to others the attachment of undue importance to their positive value of the standard free energy change ΔG^0. Furthermore, their use of conventional standard states in connexion with (**1**) can be misleading unless the thermodynamic implications are clearly recognized. This point will be taken up later.

II. The Glucose-Nitrogen Reaction

We now review the thermodynamics of the overall reaction (as used by Wilson and Burris) in which glucose as a typical nutrient is oxidized by nitrogen to give carbon dioxide and ammonia as products. The relevant standard heats (ΔH^0_f) and standard free energies (ΔG^0_f) of formation are given in Table 1, the data being taken from Latimer (1952) and from Burton and Krebs (1953) except where indicated otherwise. The conventions regarding standard states are those of Latimer (1952). Biological reactions occur under conditions well removed from the standard states, and, therefore, in Table 1 free energies of formation (ΔG_f) have been calculated for the assumed biological concentrations which are listed in column 4, and which are based on Burton and Krebs (1953). These concentrations are slightly different from those used by Wilson and Burris in equation (**1**); but it will be seen that the differences are not material. No attempt has been made to calculate values of ΔH_f for the biological concentrations; they will not differ greatly from the standard values ΔH^0_f since heats of formation are much less sensitive to concentration changes than free energies.

For the reduction of nitrogen we use one of the formal chemical half equations

$$N_2+6H^++6e^- = 2NH_3, \quad \ldots\ldots\ldots\ldots\ldots\ldots(2)$$
$$N_2+8H^++6e^- = 2NH_4^+, \quad \ldots\ldots\ldots\ldots\ldots\ldots(3)$$

and for the oxidation of glucose the formal half equation

$$C_6H_{12}O_6+6H_2O = 6CO_2+24H^++24e^-. \quad \ldots\ldots\ldots\ldots(4)$$

These combine to give the following alternative overall equations for the reduction of nitrogen by glucose:

$$C_6H_{12}O_6(\text{aq.})+4N_2(\text{gas})+6H_2O(\text{liq.}) = 6CO_2(\text{gas})+8NH_3(\text{aq.}), \quad ..(5)$$
$$C_6H_{12}O_6(\text{aq.})+4N_2(\text{gas})+6H_2O(\text{liq.})+8H^+(\text{aq.}) = 6CO_2(\text{gas})+8NH_4^+(\text{aq.}). \quad \ldots\ldots\ldots\ldots(6)$$

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* Wilson and Burris used the symbol ΔF^0 for the standard free energy change.

Under biological conditions of pH = 7, (**6**) would be a better representation than (**5**), since at this pH aqueous ammonia would be almost entirely in the form of NH_4^+. From the data in Table 1 it is found that for these reactions the standard ΔH^0 and ΔG^0, and the ΔG for the biological concentrations, are as follows:

Reaction (**5**): $\Delta H^0 = -11$ kcal; $\Delta G^0 = -59$ kcal; $\Delta G = -87$ kcal,
Reaction (**6**): $\Delta H^0 = -110$ kcal; $\Delta G^0 = -160$ kcal; $\Delta G = -112$ kcal.

For comparison with ordinary respiration, we can also derive from Table 1 the same thermodynamic quantities for the reaction

$$C_6H_{12}O_6(\text{aq.})+6O_2(\text{gas}) = 6CO_2(\text{gas})+6H_2O(\text{liq.}), \quad \ldots\ldots(7)$$

with the result that for

Reaction (**7**): $\Delta H^0 = -676$ kcal; $\Delta G^0 = -689$ kcal; $\Delta G = -690$ kcal.

TABLE 1

FREE ENERGIES AND HEATS OF FORMATION AT 25°C

Substance	Standard Heat of Formation ΔH^0_f (kcal)	Standard Free Energy of Formation ΔG^0_f (kcal)	Assumed Biological State	Free Energy of Formation in Biological State ΔG_f (kcal)
O_2(gas)	0·0	0·0	0·2 atm	−0·95
H_2(gas)	0·0	0·0		
H^+(aq.)	0·0	0·0	pH = 7	−9·52
OH^-(aq.)	—	−37·60		
H_2O(liq.)	−68·32	−56·69	Liquid	−56·69
CO_2(gas)	−94·05	−94·26	0·05 atm	−95·82
HCO_3^-(aq.)	—	−140·31		
Glucose(aq.)	−298*	−217·02	0·01M	−219·74
Pyruvate$^-$(aq.)		−113·32		
C_2H_5OH(aq.)		−42·4		
N_2(gas)	0·0	0·0	0·8 atm	−0·13
NH_3(gas)	−11·04	−3·98		
NH_3(aq.)	−19·32	−6·36	0·01M	−9·08
NH_4^+(aq.)	−31·74	−19·00	0·01M	−21·72
NH_3OH^+(aq.)		−13·54		
$N_2H_5^+$(aq.)		+21·0		
NO_2^-(aq.)		−8·25		
NO_3^-(aq.)		−26·43		

* $-\Delta H^0_f$ for glucose (aq.) calculated from data in Landolt-Börnstein (1936). Other values in this table from Latimer (1952) and Burton and Krebs (1953).

It is thus clear that the oxidation of glucose by nitrogen is a reaction which, particularly in the more probable form (**6**), involves a substantial evolution of heat and decrease in free energy, whether one considers standard states or biological concentrations. While it is true that the heat evolution and free energy decrease are considerably less than in the oxidation of glucose by oxygen, the fixation process if it occurred according to (**5**) or (**6**) would certainly require no "assistance" from any other energy-providing reaction. Although the demonstration of the thermodynamic feasibility of the reaction is no proof that biological nitrogen fixation occurs in this way, it should be remarked that nitrogen fixing organisms *do* consume nutrients such as glucose and *do* take up nitrogen, and that the simultaneous occurrence of these processes therefore makes no net demand, as far as energy is concerned, on any other metabolic process. On the contrary, the exothermic and exergonic nature of (**6**) lends thermodynamic support to the suggestion of Parker (1954) that nitrogen fixation might possibly be regarded as an alternative form of respiration with nitrogen serving as the oxidizing agent instead of oxygen.

Comparing reaction (**6**) with (**1**), the preceding paragraph shows that for (**6**) one has $\Delta G = -14$ kcal per 0·5 mole N_2, a result that is in good agreement, considering the different origin of the data and the slight differences in biological concentrations, with the value $\Delta G = -17 \cdot 5$ kcal per 0·5 mole N_2 found by Wilson and Burris (1947) for (**1**). We are thus led to consider why the latter authors obtained a *positive* standard ΔG^0 for (**1**) whereas this paper derives a substantially *negative* standard ΔG^0 for (**6**).

The reason for this discrepancy is to be found in the form of (**1**). The occurrence together of NH_4^+, OH^-, HCO_3^-, and H^+ on the right side of this equation is consistent with a valid description of a chemical system as long as these substances are at or near the concentrations given in parentheses. However, the calculation of a conventional standard free energy for (**1**) involves the consideration of a hypothetical system in which NH_4^+, OH^-, HCO_3^-, and H^+ exist simultaneously at unit concentration. Such a chemical system is of course quite unrealizable owing to the fact that NH_4OH, H_2CO_3, and H_2O are weak electrolytes. The positive ΔG^0 of reaction (**1**) thus actually includes concealed contributions from the chemical equilibria

$$CO_2+H_2O = H^++HCO_3^-;\ \Delta G^0 = +22 \cdot 27 \text{ kcal}, \qquad \ldots\ldots(\mathbf{8})$$

$$H_2O = H^++OH^-;\ \Delta G^0 = +19 \cdot 09 \text{ kcal}, \qquad \ldots\ldots\ldots\ldots(\mathbf{9})$$

whose positive standard free energies are merely the expression of the fact that the equilibrium constants of the reactions as written are very small. In fact our equation (**6**) in its standard form can be derived from equation (**1**) by subtracting 4·67 times (**8**) and 8 times (**9**). Thus we would calculate

$$\Delta G^0(\mathbf{6}) = \Delta G^0(\mathbf{1}) - 4 \cdot 67 \Delta G^0(\mathbf{8}) - 8 \Delta G^0(\mathbf{9}), \qquad \ldots\ldots\ldots(\mathbf{10})$$

which gives ΔG^0 (**6**) $= -26 \cdot 5$ kcal per 0·5 mole N_2, a result which again is in satisfactory agreement with the calculations of this paper considering the different origins of the thermodynamic data.

The analysis of the preceding paragraph shows that care must be taken in the definition of standard states where rapid chemical equilibria are involved. It also emphasizes the point, made by Gillespie, Maw, and Vernon (1953), that standard free energy changes have no biological significance apart from their relation to equilibrium constants.

III. The Use of the Frost Diagram

So far we have been concerned exclusively with the overall glucose-nitrogen reaction. However, the biological oxidation of glucose proceeds through many intermediate steps, and so presumably does the reduction of nitrogen. Ammonia may not be the first stable intermediate in the fixation process, although the evidence that it is seems very strong. Glucose or one of its intermediate oxidation products may not be the reducing agent. The object of this section is to show how the thermodynamics of a number of alternative oxidation-reduction mechanisms can be visualized readily in terms of a type of diagram proposed by Frost (1951).

Table 2

free energies of oxidation–reduction couples at 25°C

Substance	Element and Oxidation State	Oxidation–Reduction Couple Relative to Zero State	$\Delta G^0_{\frac{1}{2}}$ (kcal)	$\Delta G'_{\frac{1}{2}}$ at pH = 7 (kcal)
H_2O	O, −2	$H_2O = \frac{1}{2}O_2 + 2H^+ + 2e^-$	+56·69	+37·65
H^+	H, +1	$\frac{1}{2}H_2 = H^+ + e^-$	0·00	− 9·52
C_2H_5OH	C, −2	$C_2H_5OH + H_2O = (CH_2O)_2^* + 4H^+ + 4e^-$	+26·75	−11·33
CO_2	C, +4	$(CH_2O) + H_2O = CO_2 + 4H^+ + 4e^-$	− 1·4	−39·48
Pyruvate$^-$	C, +2/3	$(CH_2O)_3 = CH_3COCOO^- + 3H^+ + 2e^-$	− 4·8	−33·36
NH_4^+	N, −3	$NH_4^+ = \frac{1}{2}N_2 + 4H^+ + 3e^-$	+19·00	−19·08
$N_2H_5^+$	N, −2	$N_2H_5^+ = N_2 + 5H^+ + 4e^-$	−10·5	−48·58
NH_3OH^+	N, −1	$NH_3OH^+ = \frac{1}{2}N_2 + 2H^+ + H_2O + e^-$	−43·2	−52·72
NO_2^-	N, +3	$\frac{1}{2}N_2 + 2H_2O = NO_2^- + 4H^+ + 3e^-$	+105·1	+76·54
NO_3^-	N, +5	$\frac{1}{2}N_2 + 3H_2O = NO_3^- + 6H^+ + 5e^-$	+143·6	+86·48

* (CH_2O) is used as an abbreviation for $1/6(C_6H_{12}O_6)$.

An oxidation-reduction reaction can always be split formally into two half reactions or couples, and for each half reaction one can determine a standard free energy $\Delta G^0_{\frac{1}{2}}$ in terms of the conventions of Latimer (1952), which include the assignment of zero for the ΔG^0_f of H^+ and of the formal electron (e^-). Some couples and standard free energies of interest to the nitrogen problem are listed in Table 2 together with a few other well-known couples for purposes of comparison. The data are derived from the standard free energies of formation quoted in Table 1. The ΔG^0 of any complete reaction can be obtained by adding algebraically the $\Delta G^0_{\frac{1}{2}}$ values of the half reactions, provided that each $\Delta G^0_{\frac{1}{2}}$ is multiplied if necessary by a factor to ensure that the formal electrons (e^-) cancel in the addition. As pointed out previously, standard ΔG^0 values should be corrected to actual biological conditions. For the half reactions in Table 2 the most important correction concerns H^+, whose standard state is unit activity (molality), but which is at about pH = 7 under biological conditions. In the last column of Table 2 we have therefore calculated values of $\Delta G'_{\frac{1}{2}}$* for all couples for pH = 7, the other substances remaining in the standard states.

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* Burton and Krebs (1953) use $\Delta G'$ as a convenient notation for the ΔG of a reaction in which all substances except H^+ are in their standard states.

In Figure 1, the $\Delta G'_{\frac{1}{2}}$ values are plotted as recommended by Frost (1951) against oxidation number, the zero oxidation state of each element (C in glucose, O in O_2, N in N_2, H in H_2) being taken as the arbitrary reference point. In this figure there is no significance in the *absolute* positions or slopes of the lines representing the various couples; what is important is their *relative* slopes, from which one can assess rapidly the favourable or unfavourable nature of a chosen overall oxidation reaction. If, as in the inset, an oxidizing couple O and a reducing couple R_1 have the same slope, then $\Delta G' = 0$ for the reaction and the equilibrium constant $K = 1$ (at pH $= 7$). If the slope of the reducing couple (R_2) is greater than that of the oxidizing couple O, then $\Delta G'$ for the reaction is positive, $K < 1$, and the reaction could be described as "unfavourable". The reverse relation, as between R_3 and O, makes the reaction $\Delta G'$ negative and $K > 1$; the reaction could then be described as "favourable".

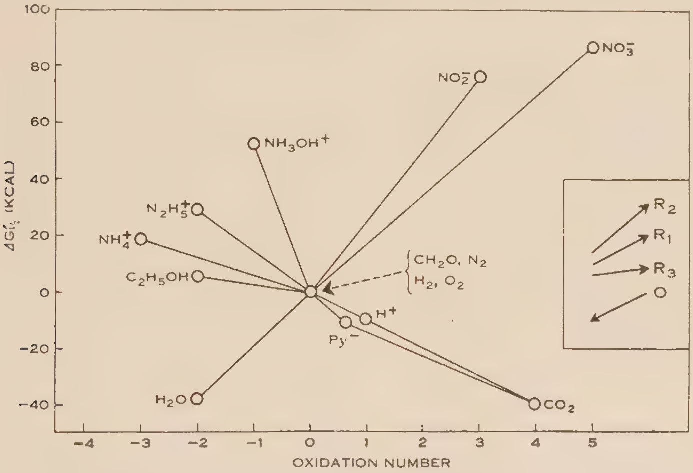

Fig. 1.—Standard free energies (with pH = 7 as the standard state for H^+) of oxidation–reduction couples plotted against the oxidation number of the element concerned.

From Figure 1 we see that the glucose–CO_2 couple (as shown in Section II) is favourable with respect to the reduction of N_2 to NH_4^+, but unfavourable for reducing N_2 to hydrazine ($N_2H_5^+$) and highly unfavourable if hydroxylamine (NH_3OH^+) is the reduction product. Taking pyruvate (Py^-) as an important intermediate in the oxidation of glucose, the Py^-–CO_2 couple is favourable with respect to N_2–NH_4^+, though somewhat less so than glucose–CO_2. The H_2–H^+ couple (at pH $= 7$) is seen to be practically equal in reducing strength to the overall glucose–CO_2 couple, a fact that is of interest in view of suggestions that hydrogenase may be associated with the fixation process (Wilson and Burris 1947; Winfield 1955*). The glucose–CO_2 and glucose–ethanol couples produce the favourable fermentation reaction—rather more favourable than the oxidation of glucose by nitrogen. The O_2–H_2O couple is of course very favourable with respect to the oxidation of glucose, just favourable with respect to the oxidation of N_2 to NO_3^-, and slightly unfavourable with respect to the oxidation of N_2 to NO_2^-. Once more it should be emphasized that these remarks apply only to thermodynamic possibilities and leave untouched the question of mechanism. Furthermore, a positive *standard* $\Delta G'$ (as defined here) does not mean that the reaction cannot proceed at all, but only that its equilibrium constant is small (<1).

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V. REFERENCES

BURK, D. (1927).—*J. Gen. Physiol.* **10**: 559.
BURTON, K., and KREBS, H. A. (1953).—*Biochem. J.* **54**: 94.
FOGG, G. E. (1955).—"New Biology." No. 18. p. 60. (Penguin Books Ltd.: Middlesex.)
FROST, A. A. (1951).—*J. Amer. Chem. Soc.* **73**: 2680.
GILLESPIE, R. J., MAW, G. A., and VERNON, C. A. (1953).—*Nature* **171**: 1147.
KALCKAR, H. M. (1941).—*Chem. Rev.* **28**: 139.
LEWIS, G. N. (1923).—"Valence and the Structure of Atoms and Molecules." (Chem. Catalog. Co.: New York.)
LATIMER, W. M. (1952).—"Oxidation Potentials." (Prentice-Hall Inc.: New York.)
LANDOLT-BÖRNSTEIN (1936).—"Physikalisch-chemische Tabellen." 5th Ed. (Springer: Berlin.)
PARKER, C. A. (1954).—*Nature* **173**: 780.
PETHICA, B. A., ROBERTS, E. R., and WINTER, E. R. S. (1954).—*Biochim. Biophys. Acta* **14**: 85.
WILSON, P. W., and BURRIS, R. H. (1947).—*Bact. Rev.* **11**: 41.
WINFIELD, M. E. (1955).—*Rev. Pure Appl. Chem.* **5**: 217.

BIOLOGICAL ACTIVITY OF COMPLEX IONS

MECHANISM OF INHIBITION OF ACETYLCHOLINESTERASE

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Summary

Previous experiments had shown that certain complex ions, which are co-ordinately saturated metal chelates lacking specific groups or centres, were toxic for mice and inhibited acetylcholinesterase. As these ions are extremely stable, undergoing no change in living organisms, it would be expected that any such effect would be physical and not chemical in nature.

Further experiments show that complex ions can inhibit acetylcholinesterase competitively and reversibly. It is suggested that the cause of this inhibition is electrostatic interaction between cationic complexes and a negatively charged site of the enzyme. The enzyme-inhibiting activity, and toxicity for mice, were found to be related to charge.

I. INTRODUCTION

Experiments on the biological activity of co-ordination complexes showed (Dwyer *et al.* 1952) that certain inorganic complexes were toxic to mice, and caused death by respiratory failure. The charged complexes inhibited acetylcholinesterase, whereas neutral complexes were inactive. The (+) and (−) forms of asymmetric complexes showed a different degree of inhibitory activity, and, in one group of complexes, different toxicities. In this paper the mechanism and the charge dependence of enzyme inhibition, and the relationship between toxic effects and anticholinesterase properties of different series of inorganic metal complexes, are examined.

II. GENERAL PROPERTIES OF COMPLEXES

The co-ordination compounds used in this work belong mainly to three series: the tris-1, 10-phenanthroline, tris-2,2′-bipyridine, and bis-2,2′,2″-terpyridine complexes with the metals Zn, Fe, Ni, Ru, and Os. The perchlorates of these cations were used. The salts themselves are completely dissociated in aqueous solutions, but the stability constants of the complex cations are high. They are co-ordinately saturated, and of great chemical stability. It can be assumed that they do not undergo any chemical change in biological systems. This has been demonstrated with $Ru(phen)_3^{++}$ ion, and details will be published elsewhere.

As none of these ions contain any specific groups or centres, their common biological activity must depend on physico-chemical properties.

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TABLE 1

LIST OF COMPLEX IONS STUDIED

Compound	Abbreviation	Reference
Tris-1, 10-phenanthroline nickel perchlorate dihydrate	$Ni\ (phen)_3\ (ClO_4)_2.2H_2O$	Blau (1898); Dwyer and Gyarfas (1949*a*); Burstall and Nyholm (1952)
Tris-5-methyl-1, 10-phenanthroline nickel perchlorate	$Ni\ (5\text{-methyl-phen})_3\ (ClO_4)_2$	Dwyer and Gyarfas (unpublished data)
Tris-5-nitro-1, 10-phenanthroline nickel chloride	$Ni\ (5\text{-nitro-phen})_3\ Cl_2$	Dwyer and Gyarfas (unpublished data)
Tris-5-chloro-1, 10-phenanthroline nickel bromide	$Ni\ (5\text{-chloro-phen})_3\ Br_2$	Dwyer and Gyarfas (unpublished data)
Tris-2, 2′-bipyridine nickel perchlorate	$Ni\ (bipy)_3\ (ClO_4)_2$	Blau (1898); Morgan and Burstall (1931)
Bis-2, 2′, 2″-terpyridine nickel perchlorate dihydrate	$Ni\ (trpy)_2\ (ClO_4)_2.2H_2O$	Morgan and Burstall (1937)
Tris-1, 10-phenanthroline ruthenium (II) perchlorate dihydrate	$Ru\ (phen)_3\ (ClO_4)_2.2H_2O$	Dwyer, Humpoletz, and Nyholm (1946); Dwyer and Gyarfas (1949*b*)
Tris-5-chloro-1, 10-phenanthroline ruthenium (II) iodide	$Ru\ (5\text{-chloro-phen})_3\ I_2$	Dwyer (1949)
Tris-5-methyl-1, 10-phenanthroline ruthenium (II) perchlorate	$Ru\ (5\text{-methyl-phen})_3\ (ClO_4)_2$	Dwyer (1949)
Tris-2, 2′-bipyridine ruthenium (II) perchlorate monohydrate	$Ru\ (bipy)_3\ (ClO_4)_2.H_2O$	Burstall (1938)
Bis-2, 2′, 2″-terpyridine ruthenium (II) perchlorate hemihydrate	$Ru\ (trpy)_2\ (ClO_4)_2{\cdot}0{\cdot}5\ H_2O$	Morgan and Burstall (1937)
Tris-1, 10-phenanthroline osmium (II) perchlorate dihydrate	$Os\ (phen)_3\ (ClO_4)_2.2H_2O$	Dwyer, Gibson, and Gyarfas (1951)
Tris-2, 2′-bipyridine osmium (II) perchlorate monohydrate	$Os\ (bipy)_3\ (ClO_4)_2.H_2O$	Burstall, Dwyer, and Gyarfas (1950)
Bis-2, 2′, 2″-terpyridine osmium (II) perchlorate trihydrate	$Os\ (trpy)_2\ (ClO_4)_2.3H_2O$	Morgan and Burstall (1937)
Tris-1, 10-phenanthroline iron (II) perchlorate monohydrate	$Fe\ (phen)_3\ (ClO_4)_2.H_2O$	Blau (1898)
Tris-2, 2′-bipyridine iron (II) perchlorate monohydrate	$Fe\ (bipy)_3\ (ClO_4)_2.H_2O$	Blau (1898)
Bis-2, 2′, 2″-terpyridine iron (II) perchlorate dihydrate	$Fe\ (trpy)_2\ (ClO_4)_2.2H_2O$	Morgan and Burstall (1937)
Tris-1, 10-phenanthroline zinc perchlorate dihydrate	$Zn\ (phen)_3\ (ClO_4)_2.2H_2O$	Jaeger and van Dijk (1934)
Tris-2, 2′-bipyridine zinc perchlorate dihydrate	$Zn\ (bipy)_3\ (ClO_4)_2.2H_2O$	Jaeger and van Dijk (1934)
Hexammine cobalt (III) chloride	$Co(NH_3)_6Cl_3$	Bjerrum and McReynolds (1946)
Nitropentammine cobalt (III) chloride	$Co(NO_2)(NH_3)_5Cl_2$	Basolo and Murmann (1953)
*trans*Dinitrotetrammine cobalt (III) chloride	$Co(NO_2)_2(NH_3)_4Cl$	Jorgensen (1894)
Trinitrotriammine cobalt (III)	$Co(NO_2)_3(NH_3)_3$	Jorgensen (1894)

It has been postulated (Pauling 1948) that the charge of such complex ions is not centred in the metallic atoms but is distributed over the complex. The distribution of this charge is determined by the electronegativity of the central metallic atom and of the ligands. The fields around the complex ion depend on the charge distribution and on the size of the ion.

With the exception of the terpyridine series (and some Co complexes listed in Table 4), the cations investigated exist in enantiomeric forms. These ions are not only sterically but also electrically asymmetric, i.e. the field around an optically active complex ion, due to the fractional distribution of charges, is not superimposable on its mirror image (Dwyer, Gyarfas, and O'Dwyer 1950).

III. Materials and Methods

(a) *Substrate*

Acetylcholine chloride (Ach) (B.D.H.) was used in 0·006M final concentration unless stated otherwise.

(b) *Enzyme*

Sheep and bovine erythrocyte acetylcholinesterase preparations were used. The former was prepared and purified by the method of Mendel and Rudney (1943). The latter was a commercial preparation (Winthrop and Stern Ltd.).

(c) *Inhibitors*

The complexes used are listed in Table 1. All samples were recrystallized.

(d) *Enzyme Activity*

The activity of acetylcholinesterase (AchE) was measured by the Warburg manometric method at 37°C, pH 7·4. Enzyme, substrate, and inhibitors were dissolved in 0·025M $NaHCO_3$. Solutions and Warburg vessels were gassed with a mixture containing 5 per cent. CO_2 in N_2. The inhibitors were incubated with the enzyme for 15 min at room temperature and for 20 min at 37°C before adding the substrate. As the rate of enzyme activity stayed always constant for the first $\frac{1}{2}$-hr of the measurement, it is expressed in terms of b_{30} (μl CO_2 evolved per 30 min).

(e) *Toxicity Estimations*

Toxicities were assessed with male albino mice from an inbred strain. Groups of eight mice were injected intraperitoneally at seven different dose rates. Solutions of complexes were made in physiological saline. The volume injected was, at each dose rate, 1·0 ml per 30·0 g of body weight. The LD_{50} values were estimated by the use of probit analysis.

IV. Experimental

(a) *Reversibility of Inhibition*

When so-called irreversible inhibitors of AchE. like di*iso*propyl fluorophosphate and tetraethyl pyrophosphate are incubated with the enzyme before the addition of the substrate, the degree of inhibition increases with increasing incubation periods.

This is taken as an indication of progressive irreversible inhibition (Augustinsson and Nachmansohn 1949). With complex cations the degree of inhibition depends only on the relative amounts of substrate and inhibitor present, and not on the incubation period. To investigate the nature of the inhibition the following experiment was carried out.

Enzyme solution (3 ml) was incubated with 4×10^{-4}M (±)-Ru(phen)$_3$(ClO$_4$)$_2$ for 3 hr at room temperature. This mixture was subsequently dialysed at 0°C for 16 hr against four changes of 100 ml of a solution containing 0·153M NaCl and 0·04M MgCl$_2$ in 0·025M NaHCO$_3$. Two controls were set up and treated in the same way. In one control the enzyme incubated with Ru(phen)$_3^{++}$ was dialysed against a 4×10^{-4}M solution of the same complex in the salt solution. The other consisted of an enzyme solution only, dialysed against the salt solution. After dialysing, 0·25 ml samples of the enzyme solution were taken, buffer and substrate were added to make a final volume of 0·8 ml, and the activity of the enzyme was compared with non-dialysed controls. It can be seen from Table 2 that the inhibition was completely reversed.

TABLE 2

REVERSION OF ENZYME INHIBITION BY DIALYSIS

Enzymic activity is expressed in μl CO$_2$/30 min (b_{30}, see text). Acetylcholine chloride concentration = 0·006M

System	Dialysed against Salt Solution	Dialysed against Salt Solution +Inhibitor	Not Dialysed
Acetylcholinesterase	105	—	99
Acetylcholinesterase+inhibitor	102	17	16

(b) *Competition Experiments*

The usual method of testing for competitive inhibition (Lineweaver and Burk 1934) involves the testing of enzyme activity in the presence of inhibitor at different substrate levels. This method did not seem satisfactory for AchE under present conditions, as the accurate determination of enzyme activity was not possible at other than near to optimal substrate levels. Therefore Augustinsson's (1948) graphical method was used, which involves the estimation of enzyme activity at various inhibitor levels and constant enzyme and substrate concentrations. In accordance with equation (1) in the case of competitive inhibition a straight line with an extrapolated intercept at "1" is obtained when the ratios of the velocities of uninhibited to inhibited enzyme activities are plotted against the inhibitor concentrations $[I]$:

$$V/V_I = 1+\frac{[I]}{K_I(1+[S]/K_m+[S]^2/K_mK_2)}, \quad \dots\dots\dots\dots(1)$$

where V represents the rate in the absence and V_I in the presence of the inhibitor, K_I the enzyme-inhibitor dissociation constant, and $[S]$ the substrate concentration.

For the commercial bovine erythrocyte cholinesterase preparation, the value of K_m was found to be $8{\cdot}7 \times 10^{-4}$, and K_2, the dissociation constant for the inactive enzyme-substrate super complex, ES_2, $2{\cdot}46 \times 10^{-2}$.

The inhibitory action of each of the complexes listed in Table 4 was estimated at least at six concentrations of the inhibitor and at 0·006M final concentration of Ach. The Ru and Os complexes of each group were also tested at 0·003M and 0·001M final Ach concentrations. The data obtained from representative experiments with six of the complexes are shown in Table 3. The plots of Figure 1 are obtained from two replicate experiments. The resulting graph illustrates the competitive nature of the inhibition.

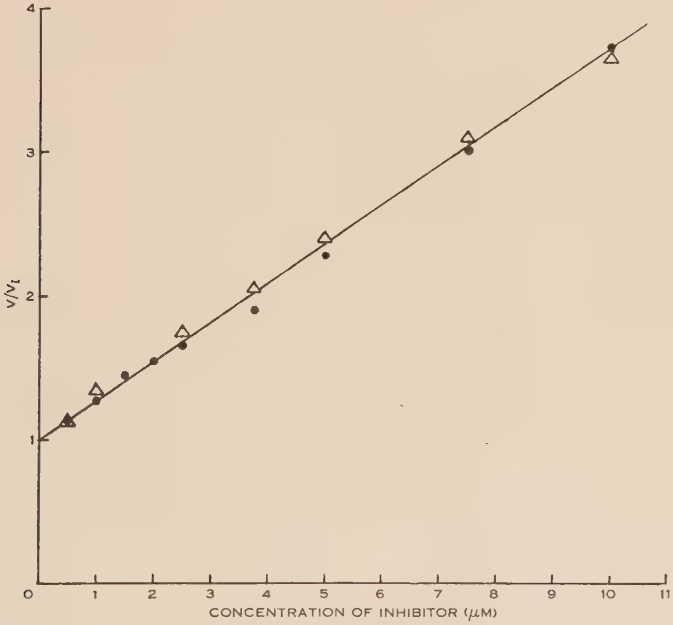

Fig. 1.—Inhibition of the enzymatic hydrolysis of acetylcholine by (±)-$Ru(phen)_3(ClO_4)_2$ as a function of inhibitor concentration. V and V_I are the reaction velocities in absence and presence of inhibitor respectively, expressed as μl CO_2 evolved per 30 min.

(c) *Charge Dependence of Inhibition*

As only charged complexes showed anticholinesterase activities (Dwyer *et al.* 1952), it was desirable to determine how these activities varied with the charge of the complexes. One possible approach is to compare the inhibitory strength of complexes which carry different amounts of total charge. Table 4 shows the inhibition obtained with such a series of complexes. Appreciable inhibition was found only with the $Co(NH_3)_4(NO_2)_2^+$ ion.

Table 5 contains five series of complex ions which all carry the same amount of total charge, but the charge distribution, and therefore the peripheral charge within the members of each series, varied. In group A this variation was achieved by introducing groups of different electrophilic capacities into the 5-position of the phenanthroline ring. Group B contains the three series of metal complexes mentioned in the Introduction. The chelating agents within each of these three series were un-

TABLE 3

INHIBITION OF ACETYLCHOLINESTERASE ACTIVITY BY COMPLEX IONS

Velocity values are expressed in terms of b_{30}. Acetylcholine chloride concentration = 0·006M

Complex	V	V/V_I					
		Relative Concentration of Complex*					
		$0{\cdot}5\times[I]$	$1{\cdot}0\times[I]$	$2{\cdot}5\times[I]$	$5{\cdot}0\times[I]$	$7{\cdot}5\times[I]$	$10{\cdot}0\times[I]$
$Zn(phen)_3^{++}$	97	1·07	1·18	1·30	1·90	2·30	2·55
$Os(phen)_3^{++}$	104	1·18	1·36	1·91	3·20	3·72	4·76
$Ni(bipy)_3^{++}$	91	1·05	1·13	1·33	1·65	1·83	2·02
$Ru(bipy)_3^{++}$	85	1·16	1·35	1·70	2·90	3·62	4·70
$Ru(trpy)_2^{++}$	96	1·09	1·18	1·50	1·81	2·37	2·68
$Os(trpy)_2^{++}$	89	1·25	1·68	2·31	3·63	4·68	6·35

* Relative concentration of complex $[I] = 10^{-6}$M for the phenanthroline complexes and 10^{-5}M for the bipyridine and terpyridine complexes. The racemates of the optically active complexes were used.

TABLE 4

INHIBITION OF ACETYLCHOLINESTERASE BY COBALT COMPLEXES

Acetylcholine chloride concentration = 0·006M

Complex*	Ionic Charge	Inhibition of Enzyme Activity (%)
$Co(NH_3)_6Cl_3$	+++	0
$Co(NH_3)_5(NO_2)Cl_2$	++	5–8
$Co(NH_3)_4(NO_2)_2Cl$	+	20
$Co(NH_3)_3(NO_2)_3$	0	0

* Owing to the limited solubilities of some of the compounds a concentration of 0·001M was used.

altered. The differences in charge distribution were caused only by the differences in electronegativity of the various central metallic atoms.

The K_I values in Table 5 were calculated from equation (1).

TABLE 5

INHIBITORY STRENGTH OF PHENANTHROLINE, BIPYRIDINE, AND TERPYRIDINE COMPLEXES*

Group A

Ion	K_I	Ion	K_I
$Ni(5\text{-nitro-phen})_3^{++}$	$24{\cdot}00 \times 10^{-7}$		
$Ni(5\text{-chloro-phen})_3^{++}$	$4{\cdot}02 \times 10^{-7}$	$Ru(5\text{-chloro-phen})_3^{++}$	$3{\cdot}83 \times 10^{-7}$
$Ni(phen)_3^{++}$	$4{\cdot}07 \times 10^{-7}$	$Ru(phen)_3^{++}$	$3{\cdot}80 \times 10^{-7}$
$Ni(5\text{-methyl-phen})_3^{++}$	$2{\cdot}12 \times 10^{-7}$	$Ru(5\text{-methyl-phen})_3^{++}$	$1{\cdot}58 \times 10^{-7}$

Group B

$Me(phen)_3^{++}$	K_I	$Me(bipy)_3^{++}$	K_I	$Me(trpy)_2^{++}$	K_I
Zn	$7{\cdot}07 \times 10^{-7}$	Zn	$14{\cdot}80 \times 10^{-6}$		
		Fe	$8{\cdot}80 \times 10^{-6}$	Fe	$14{\cdot}0 \times 10^{-6}$
Ni	$4{\cdot}07 \times 10^{-7}$	Ni	$9{\cdot}70 \times 10^{-6}$	Ni	$9{\cdot}0 \times 10^{-6}$
Ru	$3{\cdot}80 \times 10^{-7}$	Ru	$3{\cdot}03 \times 10^{-6}$	Ru	$5{\cdot}7 \times 10^{-6}$
Os	$2{\cdot}86 \times 10^{-7}$	Os	$2{\cdot}23 \times 10^{-6}$	Os	$2{\cdot}0 \times 10^{-6}$

* The phenanthroline and bipyridine complexes used were racemates.

(*d*) *Toxicity Estimations*

The signs observed after the intraperitoneal injection of the complexes were, as described earlier, hind-limb paralysis, respiratory distress, and death caused by respiratory failure (Dwyer *et al.* 1952). Physostigmine could inhibit the development of these signs (unpublished data). These facts, and the results of experiments* on frog-sartorius and rat-diaphragm preparations, suggest a curare-like activity of the complex ions. It was therefore considered of interest to compare the *in vitro* AchE inhibitory action and the toxic effects of these compounds. LD_{50} values after intraperitoneal administration were determined for the series of phenanthroline, bipyridine, and terpyridine complexes. The results of these experiments are summarized in Table 6.

V. DISCUSSION

(*a*) *Combination of the Inhibitors with the Enzyme Surface*

Both earlier experiments and those described here show that the inhibition of the enzymatic hydrolysis of acetylcholine by the complex ions is reversible and competitive. As these complexes do not contain any active groups which could block the specific centres of the enzyme, the question of their combination with [illegible] arises. The consideration of the picture of the active surface of this enzyme offers a possible explanation.

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* Experiments carried out by A. Shulman. Unpublished paper, read at the 30th meeting of A.N.Z.A.A.S., Canberra, 1954.

According to the work of various authors (Wilson and Bergmann, [illegible]; Wilson, [illegible]; Bergmann and Shimoni, [illegible]) the active surface of this enzyme protein has two distinct sites in close proximity. Centre 1 or the "anionic site" carries a single negative charge, and binds with quaternary ammonium substances by Coulombic attraction. Centre 2 or the "esteratic site" contains a basic and an acidic group. The former contributes to the binding of the substrate possibly by forming a covalent bond with the [illegible] carbon atom of [illegible]. The acidic group plays a role in the actual hydrolysis of the substrate.

Table [illegible]

[illegible] VALUES OF COMPLEXES*

Values expressed as [illegible]

[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible] [illegible]	[illegible] [illegible]	[illegible] [illegible]
[illegible]	[illegible] [illegible]	[illegible] [illegible]	[illegible] [illegible]
[illegible]	[illegible] [illegible]	[illegible] [illegible]	[illegible] [illegible]
[illegible]	[illegible] [illegible]	[illegible] [illegible]	[illegible] [illegible]

* [illegible] complexes were used. The numbers in brackets express the fiducial limits at [illegible]. [illegible]

It has been found in the above section that [illegible] competitive inhibitors of [illegible]. They inhibit by interaction with the anionic site of the enzyme. These facts and the [illegible] inhibitory action lead us to the suggestion that [illegible] of the substrate to the [illegible] of the enzyme.

[illegible] Dependence of Inhibition

[illegible] the primary importance of coulombic attraction.

[illegible]

whose charge unit, but they also vary considerably with regard to their [illegible] solubilities and [illegible] features which might well influence their ability to combine with the enzyme protein. Therefore the effect of charge on the inhibitory power of these compounds cannot be simply assessed.

The series of substituted phenanthroline complexes (group c, Table 6) are a closer approximation to the above-mentioned ideal. The introduction of an electrophilic group, such as a nitro or chloro group, increases the positive charge on the surface of the ion, while the electron-repelling methyl group decreases it. The inhibitory power of these series increases in the order $[Fe(5\text{-methyl-phen})_3]^{2+} < [Fe(5\text{-chloro-phen})_3]^{2+} < [Fe(5\text{-nitro-phen})_3]^{2+}$. This seems to indicate that the greater the peripheral charge [illegible] the stronger the inhibitory activity.

These ions do not differ very much in their size, but the size differences between them and the unsubstituted $[Fe(phen)_3]^{2+}$ ion is quite large. With the increase in [illegible] in inhibition. Such a consideration might explain the unexpectedly stronger inhibitory power of the $[Fe(5\text{-methyl-phen})_3]^{2+}$ ion compared with the $[Fe(phen)_3]^{2+}$ ion (see Table 6).

The order of the inhibitory power of the complex ions [illegible] to the [illegible] group b, Table 6 seems also to emphasize the importance of the magnitude of the peripheral charge in enzyme inhibition. The members within each of these three series differ from each other only in their different central metallic atoms. The variation in size of these complexes is negligible as it is caused only by the different ionic radii of the metals. The different electronegativities of the central metallic atoms cause the differences in charge distribution within the ions of each series: the increase in electronegativity increases the positive peripheral charge of the complex. The electronegativity increases in the order Zn < Fe < Ni < Ru < Os. With the exception of the $[Fe(phen)_3]^{2+}$ and $[Fe(bipy)_3]^{2+}$ ions the increase of the inhibitory power of the investigated complexes follows the same order in all the three series.

The K_i values for the inhibition of AChE show that the phenanthroline complexes are approximately 10 times as active as the bipyridine and terpyridine complexes. [illegible] whose [illegible] similar structure.

When comparing the inhibitory strength of these compounds the following has to be considered.

As already mentioned the $[Fe(phen)_3]^{2+}$ and $[Fe(bipy)_3]^{2+}$ ions exist in enantiomeric forms. The (+) and (−) forms of these complexes exhibit pronounced differences in their degree of inhibitory activity. The optically active forms of Zn, Ni and Fe complexes racemize rapidly, whereas those of the Ru and Os complexes are stable. When a racemic solution of the optically stable complexes is incubated with the enzyme one of the optically active forms is adsorbed preferentially as it is a stronger inhibitor. Thus the original equilibrium between the (+) and (−) forms is disturbed, the readily adsorbed form remaining in excess. The [illegible] number of ions of each of the optically active forms in the whole system (protein+complex solution) stays equal. In the Zn, Fe, and Ni complexes, racemization tends to restore the original equilibrium of the unbound forms, while those bound onto the protein do not racemize. Therefore, at the end of the binding process the whole system contains more of the stronger inhibitory form than of the weaker. Consequently the K_I values for the racemates of the optically unstable Zn, Fe, and Ni complexes reflect in reality the inhibitory power of an unequal mixture of the optically active forms, in which the stronger inhibitory component is predominant.

(c) *The Toxic Action of Complexes*

Listed in Table 6 are the LD_{50} values, which show that the toxicities of the members of the $Me(terpy)_2^{++}$ series paralleled their enzyme inhibitory power; but the degree of toxicities and anticholinesterase activities of the $Me(phen)_3^{++}$ and $Me(bipy)_3^{++}$ ions do not follow the same order. The terpyridine complexes are much more toxic than the corresponding members of the structurally related bipyridine or of the phenanthroline series. However, as the toxicity of a compound depends clearly not only on its specific action, but also on the concentration it is able to reach at its "receptor" site, the possibility of a similar mode of their action *in vivo* and *in vitro* is not excluded.

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VII. REFERENCES

AUGUSTINSSON, K. B. (1948).—*Acta Physiol. Scand.* **15** (suppl.): 52.
AUGUSTINSSON, K. B., and NACHMANSOHN, D. (1949).—*J. Biol. Chem.* **179**: 543–59.
BASOLO, F., and MURMANN, R. K. (1953).—"Inorganic Syntheses." Vol. IV. p. 174. (McGraw-Hill Book Co. Inc.: New York.)
BERGMANN, F., and SHIMONI, A. (1953).—*Biochim. Biophys. Acta* **10**: 49–54.
BJERRUM, J., and MCREYNOLDS, J. P. (1946).—"Inorganic Syntheses." Vol II. p. 217. (McGraw-Hill Book Co. Inc.: New York.)
BLAU, F. (1898).—*S. B. Akad. Wiss. Wien.* Abt. 2B **107**: 767–809.
BURSTALL, F. H. (1938).—*J. Chem. Soc.* **1938**: 1662–72.
BURSTALL, F. H., DWYER, F. P., and GYARFAS, E. C. (1950).—*J. Chem. Soc.* **1950**: 953–5.
BURSTALL, F. H., and NYHOLM, R. S. (1952).—*J. Chem. Soc.* **1952**: 3570–9.
DWYER, F. P. (1949).—*J. Roy. Soc. N.S.W.* **83**: 134–7.
DWYER, F. P., GIBSON, N. A., and GYARFAS, E. C. (1951).—*J. Roy. Soc. N.S.W.* **84**: 68–70.
DWYER, F. P., and GYARFAS, E. C. (1949*a*).—*J. Roy. Soc. N.S.W.* **83**: 232–4.
DWYER, F. P., and GYARFAS, E. C. (1949*b*).—*J. Roy. Soc. N.S.W.* **83**: 170–3.
DWYER, F. P., GYARFAS, E. C., and O'DWYER, M. F. (1950).—*Nature* **167**: 1036.
DWYER, F. P., GYARFAS, E. C., ROGERS, W. P., and KOCH, J. H. (1952).—*Nature* **170**: 190–1.
DWYER, F. P., HUMPOLETZ, J. A., and NYHOLM, R. S. (1946).—*J. Roy. Soc. N.S.W.* **80**: 212–16.
JAEGER, F. M., and VAN DIJK, J. A. (1934).—*Proc. Acad. Sci. Amst.* **37**: 284–90.
JORGENSEN, S. M. (1894).—*Z. anorg. Chem.* **5**: 147–96.
LINEWEAVER, H., and BURK, D. (1934).—*J. Amer. Chem. Soc.* **56**: 658–66.

MENDEL, B., and RUDNEY, H. (1943).—*Biochem. J.* **37**: 59–63.
MORGAN, G. T., and BURSTALL, F. H. (1931).—*J. Chem. Soc.* **1931**: 2213–18.
MORGAN, G. T., and BURSTALL, F. H. (1937).—*J. Chem. Soc.* **1937**: 1649–55.
PAULING, L. (1948).—*J. Chem. Soc.* **1948**: 1461–7.
WILSON, I. B., and BERGMANN, F. (1950).—*J. Biol. Chem.* **185**: 479–89.
WILSON, I. B. (1952).—*J. Biol. Chem.* **197**: 215–25.

THE MUTAGENIC ACTION OF FORMALDEHYDE ON *DROSOPHILA MELANOGASTER*

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Summary

(i) Formaldehyde, added to the food medium of *Drosophila melanogaster* larvae, was investigated as a mutagenic agent.

(ii) The recessive lethal mutation frequency on chromosome *II* was found to be approximately twice that on chromosome *X*.

(iii) Formaldehyde was much more mutagenic in a food medium fortified with dead yeast than in unfortified medium.

(iv) Increased formaldehyde concentration increased the lethal mutation frequency.

(v) Fortified formaldehyde medium retained its mutagenicity for some days under conditions of larval working, the actual period depending on the formaldehyde concentration.

(vi) Intensity of larval working over the range investigated had no significant effect on the mutagenicity.

(vii) Increased developmental time did not increase mutation rate.

(viii) The variation in mutagenic response obtained between different mating periods can be explained in terms of a stage (or stages) in spermatogenesis which is preferentially or exclusively sensitive to the action of formaldehyde, coupled with factors that vary the quantity or activity of mutagen reaching the chromosomes during the sensitive stage or both.

I. INTRODUCTION

Rapoport (1946) found that formalin added to the food medium of *Drosophila melanogaster* induced a high frequency of sex-linked recessive lethal mutations. The mutagenicity and mode of action of formaldehyde have since been investigated extensively by Auerbach (1949*a*, 1949*b*, 1951*a*, 1951*b*, 1952, 1953), Auerbach and Moser (1953*a*, 1953*b*), and Herskowitz (1950, 1953, 1954, 1955). The experiments described here compared the mutagenic response of chromosomes *X* and *II* to formaldehyde, and examined the effects of formaldehyde concentration, larval nutrition, and larval working of the food medium on its mutagenic efficiency.

II. CULTURE METHODS AND TECHNIQUES

Treatment of female larvae of *D. melanogaster* with formaldehyde has so far failed to induce mutations (Herskowitz 1950; Auerbach 1951*a*, 1951*b*), so tests for recessive lethals in these experiments were done on chromosomes from treated males.

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Second chromosomes were cleaned of accumulated mutations in order to obtain an estimate of mutation rate per generation comparable to that obtained for chromosome *X*.

Wild-type flies (Oregon-R-C stock) from population cages were used. The population cages and all cultures were maintained at 25±1°C, except when removed from the constant temperature room for short periods for mating and inspection. Flies in the population cages and in some of the experimental work were maintained on dead yeast fortified medium. All cultures were seeded with live yeast. Formaldehyde medium was prepared immediately before use, the measured amount of C.P. formaldehyde being thoroughly stirred in when the boiled medium cooled to about 60°C. The amount added was calculated to give the required formaldehyde concentration as volume percentage of the liquid medium.

The frequency of recessive lethal mutations was used as an index of mutation rate. The Muller-5 tester stock (Spencer and Stern 1948) was used to detect *X*-chromosome lethals. F_2 vial cultures were scored as lethals when no wild-type males were present. Vials with less than 20 flies or 10 males were discarded from the score. The CyL^4/Pm tester stock (Wallace 1951) was used to detect the second chromosome lethals. F_3 vial cultures were scored as lethals when no wild-type flies were present. Vials with less than 20 flies were discarded from the score.

III. EXPERIMENTAL PROCEDURE

(*a*) *Comparison of the Mutagenic Effects of Formaldehyde on Chromosomes* X *and* II (*Experiment* 1)

Since mutation rate varies with age of male (Muller 1945) this variate was taken into account in designing this experiment to test the effect of formaldehyde on chromosome *X* and *II*. The design of the experiment can be seen in Table 1 which shows the results.

(i) *Methods*.—The second chromosomes were cleaned using the mating system for detecting second chromosome lethals. Wild-type males selected at random from a population cage were mated to CyL^4/Pm virgin females in 10 culture bottles: five males and 10 females per bottle. When the F_1 generation had been emerging for 2 days, five CyL^4/+ males were taken at random from each bottle and individually mated in vials to three or four virgin CyL^4/Pm females. This ensured that a representative sample of genotypes was taken and that sufficient lethal-free lines would be available in the F_3 generation after those lines carrying lethals were discarded. The F_2 progeny were collected every 12 hr to obtain virgin CyL^4/+ females, which were mated to similar males from the same vial, with a minimum of two males and two females in each mating. The progeny of this mating were homozygous for a wild-type second chromosome which came from the single CyL^4/+ male mated from the F_1. Of these matings, 27 were made on fortified formaldehyde medium, and 34 on normal fortified medium. The homozygous, normal, male F_3 progeny carried lethals, arising only in this generation, which were detected in the tests described below. The F_3 flies were allowed to emerge for 12 hr and the vials then inspected. Those containing several wild-type flies were emptied. Any showing no wild-type flies were left for a further 48 hr and then scored for lethality. Of the 61 F_2 matings, 12 showed no wild-type flies in the F_3 progeny. That is, 20 per cent. of the tested chromosomes were lethal. This gives an indication of the percentage of lethal-bearing second chromosomes in the Oregon-R-C stock from the population cage. From the vials that were emptied, wild-type males (lethal-free for *X*, and *II*, but containing lethal-bearing chromosomes in their gonads) were collected 12 hr later and stored for 12 hr. Every 12 hr subsequently for 4 days, males were collected and stored for 12 hr before mating. Eighty-four males were obtained from the control series and 66 from the formaldehyde series.

Table 1

TOTAL NUMBER OF CHROMOSOMES TESTED AND PERCENTAGE LETHAL-BEARING CHROMOSOMES FOR CHROMOSOMES *II* AND *X* TESTED IN FOUR MATING PERIODS FROM BOTH CONTROL AND TREATED MALES

Mating Periods of Tested Males	Chromosome	Replications	Control Chromosomes			Formaldehyde-treated Chromosomes		
			No. of Lethals	Total No. Tested	Recessive Lethals (%)	No. of Lethals	Total No. Tested	Recessive Lethals (%)
Days 1–4	*X*	1	0	117	0	2	52	3·9
		2	0	105	0	3	60	5·0
	II	1	0	99	0	10	67	15·0
		2	0	85	0	9	63	14·3
Days 5–8	*X*	1	0	125	0	1	65	1·6
		2	0	124	0	0	68	0
	II	1	0	101	0	3	64	4·7
		2	0	101	0	9	73	12·3
Days 9–12	*X*	1	0	125	0	0	68	0
		2	0	131	0	0	68	0
	II	1	0	114	0	4	60	6·7
		2	0	101	0	4	64	6·3
Days 13–16	*X*	1	0	122	0	0	54	0
		2	0	128	0	0	69	0
	II	1	0	118	0	0	64	0
		2	0	125	0	0	63	0

All ensuing matings for the actual tests were done on normal fortified medium. One-half of each of the above male groups was mated to CyL⁴/Pm virgin females and the other half to Muller-5 virgin females. Mating intensity was three males and six females per bottle. This procedure ensured a comparable genetic and environmental background for both the *X*- and second chromosomes. At the end of 4 days, all flies were removed from the bottles, females were discarded, and the males were mated to a new lot of virgins in fresh bottles. This mating procedure was repeated twice more, giving four 4-day mating periods. Thus each male's sperm was sampled through the whole experiment. All females were stored for 3 days after collection, so that their virginity was checked before mating to the males to be tested. The broods from each of the above four mating periods were followed through the appropriate test crosses for detection of recessive lethal mutations (see Fig. 1).

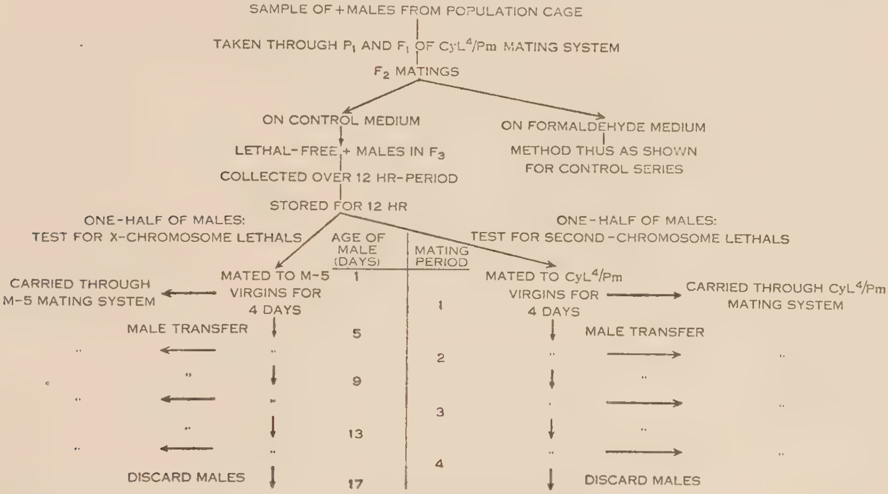
SAMPLE OF + MALES FROM POPULATION CAGE
TAKEN THROUGH P1 AND F1 OF CyL4/Pm MATING SYSTEM
F2 MATINGS
ON CONTROL MEDIUM
ON FORMALDEHYDE MEDIUM
LETHAL-FREE + MALES IN F3
METHOD THUS AS SHOWN FOR CONTROL SERIES
COLLECTED OVER 12 HR-PERIOD
STORED FOR 12 HR
ONE-HALF OF MALES: TEST FOR X-CHROMOSOME LETHALS
ONE-HALF OF MALES: TEST FOR SECOND-CHROMOSOME LETHALS
AGE OF MALE (DAYS)
MATING PERIOD
MATED TO M-5 VIRGINS FOR 4 DAYS
MATED TO CyL4/Pm VIRGINS FOR 4 DAYS
CARRIED THROUGH M-5 MATING SYSTEM
CARRIED THROUGH CyL4/Pm MATING SYSTEM
MALE TRANSFER
1
5
9
13
17
1
2
3
4
DISCARD MALES

Fig. 1.—Diagrammatic representation of experimental procedure followed in comparing the mutagenic response of chromosomes *X* and *II* to formaldehyde.

(ii) *Results*.—The numbers of chromosomes tested and the percentage lethal mutations obtained are shown in Table 1. The results demonstrate the mutagenic effectiveness of formaldehyde, particularly in the first mating period for chromosome *X*, and in the first, second, and third periods for chromosome *II*. This difference between the chromosomes in the apparent duration of their response is important. Analysis of variance showed that replications are not significantly different, and that the difference in induced mutation rates between chromosomes *X* and *II* is highly significant ($P<0{\cdot}001$).

It is generally accepted that there are about twice as many genetic loci in the second as in the *X*-chromosome. It might be expected then that the difference in mutagenic response between these chromosomes would be a function of this difference. Auerbach and Moser (1953*a*), also testing for formaldehyde-induced mutations on chromosomes *X* and *II*, tested greater numbers than in experiment 1 here. Their results (see Table 2) show about twice as many lethal mutations on the second as on the *X*-chromosome. Assuming approximately the same percentage of genetic loci on both chromosomes could mutate to lethals (Ives 1945), then the formaldehyde-induced lethal mutation rate is the same per locus for both.

This conflicts with previously-reported comparisons of mutation rates on the *X*-chromosome and the autosomes. Ives, using temperature shock (1945), or using the mutator gene high (*hi*) (1950). and Alexander (1954) using X-rays both found mutation rates per locus to be about four times as high in the autosomes as in the *X*-chromosome.

Table 2

PERCENTAGE LETHALS INDUCED BY FORMALDEHYDE IN D. MELANOGASTER. CHROMOSOMES *X* AND *II* ARE COMPARED OVER FOUR MATING PERIODS OF 3 DAYS EACH (FROM AUERBACH AND MOSER 1953*a*)

Chromosome	Mating Periods*					
	Days 0–3		Days 3–6		Days 9–12	
	No. of Chromosomes Tested	Lethals (%)	No. of Chromosomes Tested	Lethals (%)	No. of Chromosomes Tested	Lethals (%)
X	177	11·6	548	5·0	313	0·0
II	194	22·1	322	10·5	311	0·0
Ratio *X* : *II*	1 : 1·9		1 : 2·1		—	

* Chromosomes for the days 6–9 mating period were not tested.

This could be an evolutionary effect. *D. melanogaster* would have been subjected to temperature shock, mutator genes, and natural radiation, and males whose *X*-chromosomes carried lethals induced by these agencies would be lost at each generation. The *X*-chromosome thus would be expected to be less sensitive to these mutagenic agencies than would the autosomes. On the other hand, the *X*-chromosome may be no less sensitive to formaldehyde than the autosomes, as the species probably is not exposed to this mutagen in wild populations.

Table 3

PERCENTAGE LETHALS INDUCED BY FORMALDEHYDE IN D. MELANOGASTER. COMPARISON OF THE MUTAGENIC EFFICIENCY OF YEAST-FORTIFIED AND UNFORTIFIED MEDIA AT TWO CONCENTRATIONS OF FORMALDEHYDE

Medium	0·1% Formaldehyde		0·05% Formaldehyde	
	No. of Chromosomes Tested	Sex-linked Lethals (%)	No. of Chromosomes Tested	Sex-linked Lethals (%)
Fortified	112	4·5	253	4·3
Unfortified	319	0·0	433	0·9

(*b*) *Effect of Larval Nutrition on the Mutagenic Efficiency of Formaldehyde* (*Experiment* 2)

Rendel and Robertson (unpublished data) found *X*-chromosome lethal mutation rates of 2–3 per cent. on unfortified formaldehyde medium, and 6–8 per cent. on dead yeast fortified medium.

(i) *Methods*.—Sex-linked lethals were used in this and subsequent experiments as an index of mutation rate. The mutation rates on unfortified and dead yeast fortified media were compared at two formaldehyde concentrations—0·1 and 0·05 per cent. Wild-type flies were mated for 48 hr on the formaldehyde medium. Lethals were tested for in the first 4 days' sperm from treated males.

TABLE 4

TOTAL NUMBER OF *X*-CHROMOSOMES TESTED AND PERCENTAGE LETHAL-BEARING CHROMOSOMES IN FOUR MATING PERIODS FROM MALE + FLIES RAISED ON FORTIFIED FORMALDEHYDE MEDIUM

Mating Periods of Tested Males	Series	No. of Lethal *X*-Chromosomes	Total No. of *X*-Chromosomes Tested	Recessive Lethal *X*-Chromosomes (%)
Days 1–2	A	1	27	3·7
	B	3	76	3·9
	Total	4	103	3·9
Days 3–4	A	4	59	6·8
	B	2	24	8·3
	Total	6	83	7·2
Days 5–6	A	1	38	2·6
	B	1	33	3·0
	Total	2	71	2·8
Days 7–8	A	1	19	5·3
	B	0	27	0·0
	Total	1	46	2·2

(ii) *Results*.—These are tabulated in Table 3. At both concentrations, there were striking differences in the frequency of lethal mutations between fortified and unfortified media. There were no differences in the rates with different strengths of formaldehyde except possibly an increase on unfortified medium when the dose was decreased.

(*c*) *Effect of Larval Working of Formaldehyde Medium on Its Mutagenic Efficiency* (*Experiment* 3)

Auerbach (1953), Auerbach and Moser (1953*a*), and Herskowitz (1954) expected the mutagenic effectiveness of formaldehyde medium to be zero after 24 hr working by larvae. However, this effective period may be longer on fortified medium. In this preliminary experiment, the effect of 4 days working was tested, the length of the larval stage being approximately 4 days.

(i) *Methods*.—Four female and two male ebony (e^{11}) flies were mated in each of 10 bottles for 48 hr and then removed. Forty-eight hr later, four female and two male wild-type flies were mated in each bottle for 48 hr. Thus the medium had been "worked" for 4 days prior to the mating of the wild-type flies. The "period of preworking" is defined as that time interval from the mating of the e^{11} flies to the mating of the wild-type flies whose progeny are tested for lethal chromosomes. This is not the true period of larval working, but it is a convenient index. Wild-type males emerging from the fortified medium were collected at 12 hr intervals and stored for 12 hr before being mated to the Muller-5 tester stock. Those emerging in the first 36 hr (series A) and the last 48 hr (series B) were used to test for lethal-bearing X-chromosomes. Four 2-day mating periods of the males were tested, and, so that results could be compared approximately with those of experiment 1, a mating intensity of one female per male was used (Lamy 1947). The same technique as in experiment 1 was used to obtain the four sequential mating periods.

Table 5

percentage frequency of sex-linked lethal mutations induced by 0·1% formaldehyde on worked and unworked fortified medium

Medium	Mating Periods				Mean Developmental Time (days)
	Days 1–4		Days 5–8		
	No. of Chromosomes Tested	Frequency of Sex-linked Lethal Mutations (%)	No. of Chromosomes Tested	Frequency of Sex-linked Lethal Mutations	
Worked	186	5·7	117	2·7	12·8
Unworked	112	4·5	133	0·8	11·0

(ii) *Results*.—The results are tabulated in Table 4. Table 5 shows the comparison between the lethal mutation frequency produced by 0·1 per cent. formaldehyde on worked and unworked fortified medium. The results from Table 4 were converted to 4-day mating periods for comparison with the data of experiment 1. This comparison demonstrates that 4 days preworking of fortified 0·1 per cent. formaldehyde medium did not reduce its mutagenic efficiency.

(*d*) *Experiment* 4

Formaldehyde may be held in the medium in labile combination with some food constituent. More intense working of the medium might break up this combination more rapidly. Increased time of preworking with the same working intensity also may decrease the expected mutagenic response. It is possible that there are interactions between these factors and the formaldehyde concentration.

This experiment was designed and carried out by one of us (J.S.F.B.) to investigate the effect of the following three factors on the mutagenicity of the formaldehyde medium: (1) number of larvae working the medium, i.e. intensity of preworking; (2) time of preworking; (3) formaldehyde concentration.

Different preworking intensities were obtained by allowing different numbers of e^{11} females to oviposit on the formaldehyde medium. Three formaldehyde concentrations were used, viz. 0·1, 0·05, and 0·025 per cent. Within each formaldehyde concentration, the design was as shown in Table 6. The experiment included 48 treatments. 250 X-chromosomes were to be tested for lethality in each treatment.

TABLE 6

DESIGN OF EXPERIMENT 4—TESTING THE EFFECT OF LARVAL WORKING AT DIFFERENT INTENSITIES FOR DIFFERENT LENGTHS OF TIME ON THE MUTAGENIC EFFICIENCY OF FORMALDEHYDE MEDIUM

e^{11} mating intensity (No. of pairs)	0	2	6	12
Preworking period (days)	0	2, 4, 6, 8, 10	2, 4, 6, 8, 10	2, 4, 6, 8, 10

(i) *Methods.*—All matings in every generation of the testing were done on fortified medium. In every treatment, flies were mated in each of three bottles of fortified formaldehyde medium. Two males and four females were used in all wild-type matings. The initial ebony and wild-type matings were terminated after 48 hr. For example, six pairs of ebony flies were mated in each of three bottles for 48 hr. Four days after this mating was terminated, two male and four female + flies were mated for 48 hr. This treatment was then six pairs of ebony flies providing a certain preworking intensity and 6 days preworking. The e^{11} and + flies that emerged from each mating bottle were counted at 12-hr intervals up to and including the collection where sufficient + males for lethal testing had been obtained. + males were stored for 12 hr before mating to Muller-5 virgins. In this generation, for each treatment, three or four + males and at least six Muller-5 virgins were mated in each of 10 bottles. These matings were terminated at the end of 4 days. The F_1 generation was allowed to emerge for 3–4 days before the females were mated individually in vials. This meant that most females used were inseminated before being placed in vials. Vials were scored for lethality on the eleventh day after mating.

(ii) *Results.*—The number of + males that emerged each 12 hr was known so that the average developmental time for the tested males in each treatment was calculated. Total emergences from each of the three bottles per treatment had been obtained. A corrected total emergence for each treatment was calculated by weighting the emergence from each bottle by the number of tested + males obtained from that bottle. This was done to reduce variation in intensity of working between bottles of the one treatment.

TABLE 7

PERCENTAGE LETHALS, CORRECTED TOTAL EMERGENCES, AND WEIGHTED DEVELOPMENTAL TIMES OF TESTED + MALES AT THREE FORMALDEHYDE CONCENTRATIONS, WITH DIFFERENT MATING INTENSITIES AND NUMBER OF DAYS PREWORKING

Formaldehyde Concn. (%)	No. of e^{11} Pairs	Pre-working Period (days)	No. of Lethal Chromosomes	Total No. Chromosomes Tested	Lethals (%)	Corrected Total Emergence	Weighted Developmental Time (to 0·5 day)
0·025	2	2	7	284	2·5	144	9·0
		4	1	267	0·4	189	9·5
		6	0	218	0·0	221	10·0
		8	2	258	0·8	179	9·5
		10	0	280	0·0	141	10·0
	6	2	3	284	1·1	310	9·0
		4	1	271	0·4	330	10·5
		6	2	277	0·7	352	10·0
		8	0	279	0·0	264	9·0
	12	2	1	258	0·4	358	9·5
		4	1	280	0·4	363	10·0
		6	1	258	0·4	556	9·5
0·05	0	0	16	252	6·4	34	10·0
	2	2	10	251	4·0	228	10·5
		4	11	267	4·1	193	11·5
		6	1	269	0·4	239	11·5
		8	5	276	1·8	195	10·5
	6	2	17	234	7·3	326	10·5
		4	4	273	1·5	321	11·5
		6	2	273	0·7	379	12·5
		8	3	251	1·2	396	11·5
	12	2	12	276	4·4	331	11·5
		4	5	240	2·1	315	11·5
0·1	0	0	27	228	11·8	45	11·5
	2	6	9	244	3·7	118	13·5
		8	13	279	4·6	154	13·5
		10	9	270	3·3	132	13·5
	6	2	7	274	2·6	218	12·0
		8	4	266	1·5	333	13·0
		10	4	250	1·6	342	15·0
	12	6	7	259	2·7	420	16·0
		8	7	272	2·6	378	15·5
		10	10	261	3·8	424	13·5

The results are shown in Table 7. Results are presented for only 33 of the planned 48 treatments. The remaining treatments were dropped during the course of the experiment because the author was unable to carry out the required number of matings. The results show that formaldehyde retained its mutagenicity for more than 24 hr, that the mutagenicity decreased gradually with increased time of working, and that the length of the mutagenic period appeared to depend on the initial concentration of formaldehyde. Within formaldehyde concentrations, the mutagenic response was not significantly affected by different intensities of preworking. Thus the lethal mutation frequency data may be regrouped as shown in Table 8. The data for the three "missing plots" were fitted by regression analysis.

TABLE 8

GROUPED DATA OF LETHAL MUTATION FREQUENCIES INDUCED BY THREE FORMALDEHYDE CONCENTRATIONS WITH DIFFERENT PERIODS OF PREWORKING OF THE MEDIUM

Data	Formaldehyde Concn. (%)	Preworking Period (days)					
		0	2	4	6	8	10
No. of lethals/total tested	0·025	—	11/826	3/878	3/753	2/537	0/280
Percentage lethals		—	1·332	0·367	0·398	0·372	0·000
Angle response		6·520	6·497	2·092	2·269	2·120	0·000
No. of lethals/total tested	0·05	16/252	39/761	20/780	3/542	8/527	—
Percentage lethals		6·400	5·125	2·564	0·554	1·518	—
Angle response		14·640	13·063	9·172	3·158	6·943	1·610
No. of lethals/total tested	0·1	27/228	7/274	—	16/503	24/817	23/781
Percentage lethals		11·800	2·555	—	3·181	2·938	2·945
Angle response		20·120	9·155	12·600	10·272	9·882	9·896

The results show distinct differences in mutation frequency between concentrations over the range tested. With increased time of preworking, the mutation frequency decreased. Interactions are suggested by a change in this trend to increased response on the sixth, eighth, and tenth days respectively for increasing concentrations. This could be quite spurious, but the fact that it occurred later with increasing concentration indicates some release of formaldehyde or a mutagenic derivative after some working of the medium, or that changes in the constitution of the medium, known to take place as the yeast grows (Sang 1949*b*), may have interacted with the formaldehyde to increase mutation rates. Differences in lethal mutation frequency between concentrations were significant and linear. Differences between days of preworking were significant and linear, with a regression coefficient (b) of $-0{\cdot}983$, where b is change in angle response per day of preworking. The results and the fitted regression lines are shown in Figure 2. The possible effect, noted above, of increased response after the sixth day of preworking was not shown by this analysis. Figure 2 clearly shows the relation between formaldehyde concentration and working of the medium in their effect on mutagenicity. Increased concentration did not alter the rate of change in the mutagenicity of the medium. Over the range tested, it caused the initial response to be higher, and with working, this mutagenicity took longer to decline to zero.

The ineffectiveness of intensity of working was unexpected. The number of emerging adults is not a good indication of the number of larvae originally present (Sang 1949*a*). Therefore, from the emergence data, we were unable to estimate the actual numbers of larvae working the medium. However, increasing the number of colony pairs did result in larger emergences so that different working intensities were obtained. If more larvae work the medium for a certain time, it seems possible that there may be a more rapid decrease in the mutagenicity of that medium. The lethal mutation frequencies in Table 7 for 0·025 per cent. formaldehyde suggest some effect of intensity. Apparently, at higher concentrations, the numbers of larvae working the medium were insufficient to deplete the formaldehyde present. The ineffectiveness of the intensity of working suggests a further possibility. There is no evidence that the decreased mutagenicity of the medium with increased time of larval working is not just an effect of aging of the food. The rate of decrease would be dependent solely on the initial formaldehyde concentration. This possibility must be tested.

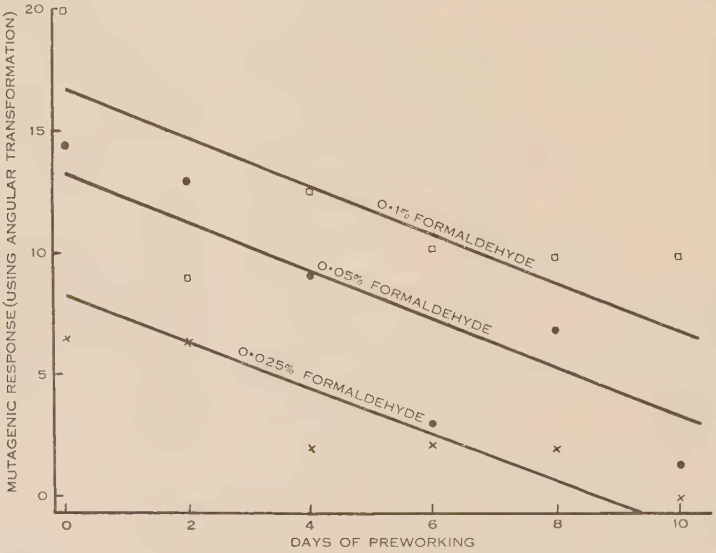

Fig. 2.—Results (transformed to angle response) of experiment 4—investigating the effect of larval working of formaldehyde medium on its mutagenic efficiency—and the fitted regression lines.

IV. Discussion

(*a*) *Variation in Mutagenic Response with Mating Period*

The negative regression of induced lethal mutation rate on mating period obtained in experiment 1 (Table 1), with four mating periods each of 4 days duration, is similar to the regressions reported by Auerbach (1951*b*) with two mating periods of 5 days each, and by Auerbach and Moser (1953*a*) with three periods of 3 days each

(Table 2). Analysis of the results of experiment 3 (Table 4) revealed no significant regression over four periods of 2 days each. Plotting the results (see Fig. 3) suggests a quadratic regression curve with a maximum in the second mating period. The accurate detection of the mutagenic response pattern of sperm samples from sequential mating periods requires a finer partitioning of the mating period and the testing of larger chromosome numbers.

However, the fact that there is a difference in mutation frequency between sequential mating periods of the same tested males indicates that different batches of sperm have been subjected to different mutagenic conditions. There may be a stage (or stages) in spermatogenesis that is preferentially or exclusively sensitive to the mutagen. This sensitive stage hypothesis is supported by the data of Auerbach and Moser (1953*a*), where delaying the exposure of larvae to the formaldehyde medium until later periods of development led to the occurrence of a maximum mutagenic response in the later mating periods. That is, sperm that had passed the sensitive stage early in larval development were not affected.

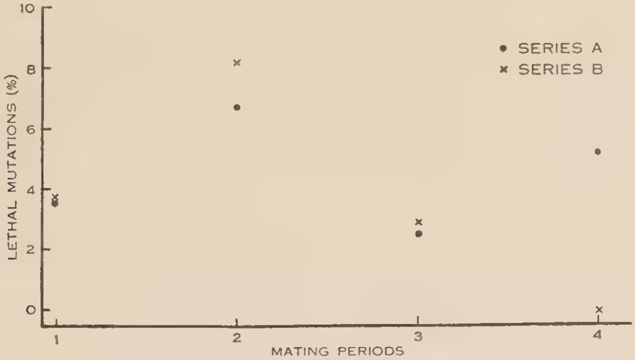

Fig. 3.—Per cent. lethal mutations induced by formaldehyde in worked fortified medium over four sequential mating periods of 2 days each.

(*b*) *Nature of the Sensitive Stage*

Adult males are not sensitive to formaldehyde supplied in the medium, even though it reaches the testis (Kaplan, unpublished data, quoted by Auerbach and Moser 1953*a*). This means that the sensitive stage occurs in the larva or pupa. There is good evidence that natural mutation rate is proportional to the number of cell divisions rather than to absolute time (Haldane 1948), indicating that errors in gene reproduction are restricted to a specific stage of the division cycle. It is possible that the chromosomes are sensitive at this stage for induced mutations as well as for natural mutations. This stage could occur when the nuclear membrane dissolves during prophase, presumably facilitating the penetration of the mutagen to the chromosomes. The absence of groups of allelic lethals from a male's sperm sample (Auerbach 1951*a*, 1951*b*) means that the sensitive stage occurs late in spermatogenesis. It could be some stage of meiosis. This is supported by the absence of a mutagenic effect in female larvae. Meiosis occurs in the egg after it is laid (Demerec 1950), and eggs have responded mutagenically to formaldehyde (Rapoport 1947; Auerbach 1952).

The germ-line sensitivity need not necessarily reside at the nucleus or chromosome level. It could be an effect of changes in the permeability of the cell membrane or cytoplasm of the germ line as spermatogenesis proceeds. This is suggested by Auerbach's work (1952, 1953) where injections of aqueous solutions of formaldehyde into the abdominal cavity of the adult male induced mutations, most of which traced back to mature sperm. Yet adult males fed on formaldehyde gave no mutagenic response in their sperm, even though the formaldehyde penetrated to the testis (see above). Therefore, there may be differences in penetration to the chromosomes, which may depend on concentration. Injected formaldehyde may be expected to be at a higher concentration around the gonad cells than is ingested formaldehyde. A comparatively high formaldehyde concentration may be necessary before any can penetrate to the chromosomes in mature sperm. This resistance to penetration may increase as spermatogenesis proceeds.

It appears that germ-line sensitivity is rather complex and that there may be more than one stage of sensitivity in spermatogenesis. The stage of maximum penetration could be controlled by an interaction between two or more sensitive stages at different sites (e.g. cell membrane permeability and nuclear membrane permeability). As the time of sensitivity at each site need not necessarily be correlated, maximum penetration of the mutagen would occur where the times of sensitivity for each site had their maximum overlap during spermatogenesis. If the sensitive stages occurred at the same site, e.g. at the nuclear membrane, the pattern of mutagenic response could have as many maxima as there are sensitive stages.

(c) *Factors Affecting the Quantity or Activity of the Mutagen Reaching Sperm Chromosomes During a Sensitive Stage*

The existence of a sensitive stage alone is insufficient to account for the variation in mutagenic response obtained in experiment 1, for all sperm would pass through a sensitive stage or stages and therefore should have equal opportunities to be affected by the mutagen. However, as spermatogenesis proceeds throughout the life of the fly, the sensitive stage or stages would be expected to occur at different developmental periods for different sperm. Sperm used first are presumably those which mature first. If this is so, sperm tested in the first mating period had their sensitive stage or stages earlier than sperm tested in the second mating period and so on. The following factors are proposed to explain the absence of a mutagenic response from sperm tested in the later mating periods:

(i) Only larvae ingested the formaldehyde medium, as adults were removed from the medium within 12 hr of emergence. Further it is unlikely that an active substance like formaldehyde would remain unmetabolized. If so, when the larvae cease feeding in order to pupate, formaldehyde or its mutagenic derivative would soon be removed or broken down, so that only those sperm which were sensitive during the larval stage would be affected by the mutagen.

(ii) As the medium ages and is worked by the larvae, its mutagenic efficiency decreases (see experiment 4). Thus as the larvae age, they ingest less mutagenic substance. Sperm that pass through a sensitive stage late in larval life would be exposed to less mutagen than sperm that matured earlier.

(iii) Elimination or detoxication mechanisms may develop as a direct response to the presence of formaldehyde. This hypothesis is suggested by the results of Auerbach and Moser (1953*a*). They took freshly-emerged, 1-day, 2-day, and 3-day old larvae from normal medium and placed them on formaldehyde medium to complete their development. They used formaldehyde concentrations of 0·18–0·25 per cent. (expressed as a percentage of the volume of water used in medium preparation). 0·18 per cent. is equivalent to 0·1 per cent. on the standard used here. Therefore, the minimum concentration used by Auerbach and Moser should have been mutagenic for at least 10 days (experiment 4). Results of comparable experiments are shown in Table 9. In their experiment FF29 (Table 9), the mutagenic response obtained in the second mating period for larvae placed on the formaldehyde medium on the second day of their larval life is almost double the response, for the same mating period, obtained from larvae placed on the formaldehyde media on the first day of their larval life. Also responses were obtained in the third and fourth mating periods

TABLE 9

RESULTS OF COMPARABLE EXPERIMENTS OF AUERBACH AND MOSER (1953*a*) AND THE AVERAGED RESULTS OVER FOUR MATING PERIODS FOR EACH OF THEIR FOUR TREATMENTS

Treatment No. and Day on which Treatment Given	Experiment No.	Mating Periods (3 days each)			
		1	2	3	4
1. First day of larval life	FF29	5·7	1·7	0	0
	FF39A	5·5	1·5		
	FF45F	7·7	2·5		
	FF17	8·2	2·0		
	Average	6·8	1·9	0	0
2. Second day of larval life	FF29	1·6	3·1	1·4	0·4
	FF9B	5·5	0		
	FF41·0	12·3	3·0		
	FF17F	5·1	0·8	0·4	0
	Average	6·1	1·7	0·9	0·2
3. Third day of larval life	FF29	1·2	1·2	1·6	1·3
	FF39	0·6	1·2	1·3	
	FF22	0·2	2·1	0	0
	Average	0·7	1·5	1·0	0·7
4. Fourth day of larval life	FF29	0·7	0·6	1·1	0·9
	FF39	0·3	4·1	0	
	FF22	0·6	3·6	0·2	0
	Average	0·5	2·8	0·4	0·5

for larvae transferred to the formaldehyde medium on the second, third, and fourth days of their life, whilst no response was obtained in these mating periods for those larvae which spent all their development in contact with the formaldehyde media. The fact that larvae exposed at later stages of their development showed higher responses in the later mating periods, even though they spend less time in contact with the mutagen, makes these increased responses all the more striking. However, comparing these different treatments of Auerbach and Moser, the decline in mutagenicity of the medium must be considered. For example, sperm sensitive during the fourth day of larval life would be subjected to a higher formaldehyde concentration in treatment 4 (see Table 9), than in treatment 1. The possible relation between formaldehyde concentration and penetration to the chromosomes has been considered above.

There are other factors that could lead to variation between different batches of sperm in the quantity or activity of the mutagen reaching the chromosomes during a sperm's sensitive stage. Such factors are changes in the physiology of the fly as it develops from egg to adult leading to variations in the penetrability of the mutagen to the testis, variations in the length of the sensitive stage or stages, and changes in the relative positions in time of sensitive stages at different sites.

There is an explanation which does not involve the concept of a sensitive stage. This is that sperm bearing lethal mutations are selectively eliminated from the sperm sample, and so lead to a decreasing frequency of lethal mutations in later used sperm. This hypothesis fails to account for the occurrence of the maximum mutagenic response in later mating periods when larvae are exposed to the mutagen in their late development, and is contrary to all experience with X-irradiation.

(*d*) *Effect of Developmental Time on Mutagenic Response*

Muller (1945) and Auerbach (1949*a*) expect an increase in developmental time to increase the frequency of lethal mutations obtained, because of prolongation of the "sensitive period". Increased time of preworking (Table 7) is associated with a decrease in lethal mutation frequency and an increase in developmental time. This does not mean that increased developmental time causes lower mutation rates, but in this experiment the two are correlated.

The results of experiment 3 (Table 4) show no significant difference in mutation frequency between series A and B. The mean difference in developmental time between the series was 2·4 days. It is possible that rate of spermatogenesis is largely independent of overall developmental rate.

(*e*) *Effect of Larval Nutrition on Mutagenic Response*

In experiment 2, 0·1 per cent. formaldehyde in unfortified media suppressed yeast growth, and no working of the medium by larvae was observed. In some of these cultures, mould developed from the tenth day after mating. After a further 10 days, flies started to emerge from the mould-contaminated bottles only. The failure of flies to emerge before the development of the mould was probably due to starvation, since growth of yeast cells was suppressed by the formaldehyde. Flies emerged in large numbers where dead yeast was supplied in quantities sufficient to support growth. 0·05 per cent. formaldehyde in unfortified medium was not at a concentration which suppressed yeast development completely, and working of the medium was observed. On 0·1 per cent. formaldehyde medium, the larvae apparently fed on fungus alone. In this group, there was no mutagenic response. This could be due to one of the following factors:

(i) The formaldehyde may have evaporated or been lost from the medium before larvae emerged onto it.

(ii) The mould did not absorb formaldehyde or its mutagenic derivative.

(iii) The mould's metabolism converted formaldehyde to a non-mutagenic form. Handler, Bernheim, and Klein (1941), Mackenzie and du Vigneaud (1949), and Paretsky and Werkman (1950), (all quoted in Engelsberg 1952) consider that the metabolism of some microorganisms has formaldehyde as an intermediary in the synthesis of glycine and sarcosine.

The fact that no mutations occurred on the unfortified 0·1 per cent. formaldehyde medium may be indicative of the mode of ingestion of the mutagen. If the larvae feed almost entirely on live moulds or yeast, perhaps little or no formaldehyde would be ingested. However, if other food constituents, such as dead cells, were included in their diet, some formaldehyde may be ingested with these other food constituents. When the medium is fortified with dead yeast and a high concentration of formaldehyde is used, the larvae eat dead cells only and thus probably ingest considerable quantities of formaldehyde so that high mutation rates result.

It appears that variations in the mutagenic response with different cultural conditions depend on the quantity of formaldehyde or its mutagenic derivative absorbed in the ingested food. Highest mutation rates were obtained on dead yeast fortified medium: probably because the larvae lived almost exclusively on dead material which probably carried a large amount of absorbed formaldehyde.

V. CONCLUSIONS

(i) Formaldehyde-induced lethal mutation rate is the same per locus for both the *X*- and second chromosomes.

(ii) Dead yeast fortified formaldehyde medium is considerably more mutagenic than corresponding unfortified medium. The variation in mutagenic response with different cultural conditions is postulated to be a function of the quantity of formaldehyde or its mutagenic derivative absorbed in the ingested food.

(iii) Induced lethal mutation frequency increases with formaldehyde concentration.

(iv) Fortified formaldehyde medium retains its mutagenicity for some days under conditions of larval working, the actual period depending on the formaldehyde concentration.

(v) Intensity of larval working over the range investigated has no significant effect on the mutagenicity.

(vi) The negative regression of induced lethal mutations on mating period needs further investigation, using mating periods of 1 day or less and testing larger chromosome numbers.

(vii) The variation in mutagenic response obtained between different mating periods has been explained in terms of a stage (or stages) in spermatogenesis which is preferentially or exclusively sensitive to the action of formaldehyde, coupled with factors which vary the quantity or activity of mutagen reaching the chromosomes during a sensitive stage or both.

(viii) The expectation of Muller (1945) and Auerbach (1949*a*) that increased developmental time would increase mutation rate apparently is not realized.

VI. Acknowledgments

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VII. References

Alexander, M. L. (1954).—Mutation rates at specific autosomal loci in the mature and immature germ cells of *Drosophila melanogaster*. *Genetics* **39**: 409–28.

Auerbach, C. (1949*a*).—Chemical mutagenesis. *Biol. Rev.* **24**: 355–91.

Auerbach, C. (1949*b*).—The mutagenic mode of action of formalin. *Science* **110**: 419–20.

Auerbach, C. (1951*a*).—Problems in chemical mutagenesis. *Cold Spr. Harb. Symp. Quant. Biol.* **16**: 199–213.

Auerbach, C. (1951*b*).—Some recent results with chemical mutagens. *Hereditas, Lund.* **37**: 1–16.

Auerbach, C. (1952).—Mutation tests on *Drosophila melanogaster* with aqueous solutions of formaldehyde. *Amer. Nat.* **86**: 330–2.

Auerbach, C. (1953).—Sensitivity of *Drosophila* germ cells to mutagens. *Heredity* **6** (Suppl.): 247–57.

Auerbach, C., and Moser, H. (1953*a*).—An analysis of the mutagenic action of formaldehyde food. I. Sensitivity of *Drosophila* germ cells. *Z. indukt. Abstamm.-u. VererbLehre* **85**: 479–504.

Auerbach, C., and Moser, H. (1953*b*).—An analysis of the mutagenic action of formaldehyde food. II. The mutagenic potentialities of the treatment. *Z. indukt. Abstamm.-u. Vererb-Lehre* **85**: 547–63.

Demerec, M. (1950).—"Biology of *Drosophila*." (John Wiley & Sons Inc.: New York.)

Engelsberg, E. (1952).—Mutagenic action of formalin on bacteria. *J. Bact.* **63**: 1–11.

Haldane, J. B. S. (1948).—Croonian Lecture: The formal genetics of man. *Proc. Roy. Soc.* B **135**: 147–70.

Handler, P., Bernheim, M. L. C., and Klein, J. R. (1941).—The oxidative demethylation of sarcosine to glycine. *J. Biol. Chem.* **138**: 211–18.

Herskowitz, I. H. (1950).—The differential induction of lethal mutations by formalin in the two sexes of *Drosophila*. *Science* **112**: 302-3.

Herskowitz, I. H. (1953).—Formaldehyde-induced mutation in mature sperm and early developmental stages of *D. melanogaster*. *Genetics* **38**(6): 668 (Abstr.).

Herskowitz, I. H. (1954).—The mutagenicity of formaldehyde in different early developmental stages of *D. melanogaster* males. *Amer. Nat.* **88**: 45–9.

Herskowitz, I. H. (1955).—The production of mutations in *Drosphila melanogaster* with chemical substances administered in sperm baths and vaginal douches. *Genetics* **40**(1): 76–89.

Ives, P. T. (1945).—The genetic structure of American populations of *Drosophila melanogaster*. *Genetics* **30**: 167–96.

IVES, P. T. (1950).—The importance of mutation rate genes in evolution. *Evolution* **4**: 236–52.

LAMY, R. (1947). —Observed spontaneous mutation rates in relation to experimental technique. *J. Genet.* **48**: 223–36.

MACKENZIE, C. G., and DU VIGNEAUD, V. (1949).—Formation of formaldehyde in the biological oxidation of the methyl group to sarcosine. *Fed. Proc.* **8**: 223.

MULLER, H. J. (1945).—Age in relation to frequency of spontaneous mutations in *Drosophila*. *Yearb. Amer. Phil. Soc.* **1945**: 150-3.

PARETSKY, D., and WERKMAN, L. H. (1950).—The bacterial metabolism of glycine. *Arch. Biochem.* **25**: 288–98.

RAPOPORT, J. A. (1946).—Carbonyl compounds and the chemical mechanism of mutations. *C.R. Acad. Sci. U.R.S.S.* **54**: 65–7.

RAPOPORT, J. A. (1947).—Chemical reaction with the protein amino groups in gene structure. *J. Gen. Biol., Moscow* **8**: 359–79. (In Russian.)

SANG, J. H. (1949*a*).—The ecological determinants of population growth in a *Drosophila* culture. V. The adult population count. *Physiol. Zoöl.* **22**: 210–23.

SANG, J. H. (1949*b*).—The ecological determinants of population growth in a *Drosophila* culture. VI. The total population count. *Physiol. Zoöl.* **22**: 223–35.

SPENCER, W. P., and STERN, C. (1948).—Experiments to test the validity of the linear R-dose/mutation frequency relationship in *Drosophila* at low dosage. *Genetics* **33**: 43–74.

WALLACE, B. (1951).—Genetic changes within populations after X-irradiation. *Genetics* **36**: 612–28.

CHANGES IN THE FREE AMINO ACIDS OF THE BLOOD OF BLOWFLY LARVAE AT METAMORPHOSIS

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Summary

Blood from the larval, prepupal, and early pupal stages of *Calliphora augur* (F.) contains the same 18 free amino acids. In addition, hydroxyproline is present in larval and prepupal blood. The quantitative differences in the concentrations of these amino acids are reported. Larval blood has the highest free amino acid content (6·6 mg/ml) followed by early pupal blood (4·6 mg/ml) and prepupal blood (3·3 mg/ml). As the larva matures to the prepupa, the greatest decreases occur in the concentrations in the blood of alanine, glutamic acid, glutamine, glycine, proline, and tyrosine. The metabolic significance of these changes are discussed.

I. Introduction

The free amino acid content of the blood of insects is very high in comparison with that of other animals, and from 5 to 20 mg/ml are commonly found. Until the advent of paper chromatography few of the amino acids had been identified, but since about 1949 knowledge of the amino acids in the blood has begun to accumulate more rapidly. Even so, very little has been published on the qualitative and quantitative differences in the amino acid composition of the blood of insects at different times during their life-cycle. Reference can be made to the work of Auclair (1953) on *Galleria mellonella*, Sarlet, Duchateau, and Florkin (1952) on *Bombyx mori* (L.), and Drilhon (1952) on *Macrothylacea rubi* L., although there are other papers in which changes in the composition of only one or two amino acids are given.

In the absence of confining blood vessels the blood of insects is free to flow among the organs and tissues within the body cavity. The blood is the reservoir for the products required for and produced by nearly every physiological activity of the insect body, and in growing or metamorphosing insects the changes in blood composition reflect the morphogenetic and biochemical transformations taking place in the tissues. Just prior to pupation, the larval cuticle of blowflies increases considerably in thickness. In the early pupal stage this cuticle is hardened and becomes dark in colour. Changes in the free amino acids and phenols of the blood at metamorphosis may therefore lead to an understanding of the nature of these processes.

II. Experimental

(a) Preparation of Samples

A culture of *Calliphora augur* (F.) was established from adults collected in the field. The larvae were reared on sheep's liver in a room maintained at 25°C. Twenty-four hours prior to the collection of larvae for experimental purposes the adult flies were deprived of liver. Liver was then placed in the cages and the larvae (approx.

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2000) laid over a period of 30 min were collected. These larvae were provided at all times with an abundant supply of liver. After 72 hr the larvae were almost fully grown but were still actively feeding. By the time the larvae were 96 hr old they had left their food, had voided their gut contents, and entered what may be called the prepupal stage. 2–3 days later the larva becomes immobile and shortly afterwards hardens and darkens to form the puparium. Blood samples were taken from the almost fully grown feeding larvae (72 hr old) (in future called larval blood), from the prepupae (96 hr old), and from early pupae. For the latter, immobile individuals were selected in which the puparium was not coloured or had only just begun to colour.

Before blood samples were collected, the various stages were washed in several changes of distilled water and dried on filter paper. Blood was collected by piercing the anterior end of the animal with a needle and collecting, on the end of a small spatula, the drop of blood which appeared. The blood was placed immediately under light petroleum (boiling range 40–60°C) to prevent it darkening. Approximately 2 ml of blood was collected from 200 larvae or 200 prepupae but considerably less blood was obtained from the early pupal stage. A convenient tool to use when collecting the blood samples was a length of stainless steel wire ($\frac{1}{16}$ in. dia.) sharpened to a needle point at one end and flattened to form a small spatula at the other end. Although by this method it was not possible to collect all the blood from each animal, one could be sure that the blood which was collected was uncontaminated by other tissues.

Each of the samples of blood was treated as follows: the blood, under light petroleum, was centrifuged, and a known volume removed and mixed with an equal volume of 10 per cent. trichloroacetic acid to precipitate the proteins. After 1 hr the suspension was centrifuged and the supernatant poured into a stoppered tube. The precipitate was washed with three lots of 2 ml of 5 per cent. trichloroacetic acid when the extract no longer gave a positive ninhydrin test for amino acids. The combined washings and original supernatant were freed from trichloroacetic acid by repeated extraction with ether. The aqueous solution was evaporated to dryness *in vacuo* over phosphorus pentoxide at room temperature and the residue dissolved in phosphate buffer of pH 7 (0·04M $Na_2HPO_4 \cdot 2H_2O$ and 0·027M KH_2PO_4) containing 10 per cent. *iso*propanol. The volume of buffer used was equal to the original volume of blood taken. Hydrogen peroxide (100 vol., one-fifth of the volume of the sample taken) was added to a sample of each protein-free specimen of "blood", the solution allowed to stand for several hours, evaporated to dryness *in vacuo*, and the residue taken up in a volume of buffer equal to that of the sample taken. This treatment converted any cystine or cysteine to cysteic acid and any methionine to methionine sulphone.

(*b*) *Paper Chromatography*

(i) *Qualitative Analysis of Amino Acids.*—The six solutions representing the three samples of blood were subjected to qualitative paper chromatography using both the one- and two-dimensional methods. The following solvents were used for development of the chromatograms: 75 per cent. aqueous phenol; phenol buffered to pH 10 (74 per cent. phenol, 26 per cent. buffer (0·053M boric acid and potassium chloride.

0·047M sodium hydroxide)); 60 per cent. aqueous acetone; acetone buffered to pH 7 (60 per cent. acetone and 40 per cent. buffer (0·04M $Na_2HPO_4.2H_2O$ and 0·027M KH_2PO_4)); 65 per cent. aqueous pyridine; and *n*-butanol–acetic acid–water (77:6:17 by volume). Where buffer solutions were used the paper was treated with the same buffer solution before use. The buffer solution was allowed to flow on to the paper from a pipette until the paper was saturated. The paper was air dried before use. For two-dimensional runs various combinations of these solvents were used in order to obtain satisfactory separation of all amino acids. In addition to ninhydrin the following special reagents were used to detect the spots corresponding to the amino acids: for proline and hydroxyproline—isatin (Hackman and Lazarus 1956); for histidine—diazotized sulphanilamide (Hackman and Lazarus 1956); and for taurine—*o*-phthaldehyde (Curzon and Giltrow 1954). Hydrolysis (6N HCl in a sealed tube at 110°C for 24 hr) of the deproteinized blood samples did not produce any new compounds.

(ii) *Quantitative Analysis of Amino Acids.*—The amino acids present in the six solutions representing the samples of blood were estimated by the quantitative paper chromatographic method of Hackman and Lazarus (1956) using five replicates. The samples which had been treated with hydrogen peroxide were used for the phenol solvent, the other samples for the remaining three solvents.

(iii) *Phenols.*—The samples of blood were examined for phenolic compounds. Benzene–acetic acid–water (40:40:20 by volume), chloroform–acetic acid–water (50:25:25 by volume), *n*-butanol–phenol–water (1:9:10 by weight), and an aqueous solution of carbon dioxide of pH 4·2 were used as solvent systems for development of the paper chromatograms. To detect spots due to phenols the following reagents were used: Folin-Denis reagent, Folin's reagent, Millon's reagent, and Pauly's reagent (Dalgliesh 1952) and ferric chloride and James' ferricyanide reagent (James 1948; James and Kilbey 1950). No phenolic compounds other than tyrosine were detected.

III. Results and Discussion

The results of the qualitative and quantitative analysis of the free amino acids present in the blood of *C. augur* at three different stages of its life-cycle are given in Table 1. By "trace" is meant an amount which was too low to be estimated conveniently even after concentrating the solutions or by multiple spotting (not greater than 0·1 mM). Tryptophan, histidine, β-alanine, ornithine, and β-(3,4-dihydroxyphenyl)alanine could not be detected in any of the blood samples. A single sample of blood from some 200 individuals of each stage was used for the analysis. However, many qualitative analyses (paper chromatography) have been performed on different samples of blood corresponding to each stage and in every case the same amino acids were detected and, as far as could be judged, the variations in amounts present were similar.

Blood from the larval, prepupal, and early pupal stages contains the same 18 free amino acids. In addition hydroxyproline is present in larval and prepupal blood. The free amino acid content of the larval blood amounts to about 6·6 mg/ml, of prepupal blood 3·3 mg/ml, and of early pupal blood 4·6 mg/ml. Considering larval and prepupal bloods only, the greatest decreases occur in the concentrations of alanine, glutamic acid, glutamine, glycine, proline, and tyrosine. Although the concentration of aspartic acid in prepupal blood is only half that in larval blood the concentrations are very low. During the pupal stage, larval tissues are being broken down and the adult fly is being formed. Consequently it would be very difficult to correlate changes in amino acid content with any particular metabolic process.

Amino acids serve as units for the synthesis of proteins or they may be metabolized. They must make some contribution to the osmotic pressure of the blood and they must have some buffer action even if only a minor one. An early step in the breakdown of amino acids is the loss of the amino groups which may be stored, transferred to a suitable compound (transamination), or excreted as ammonia, urea, or uric acid. Amino groups are stored by reaction with glutamic acid and aspartic acid to form glutamine and asparagine respectively. The amino acids principally concerned in transamination reactions are alanine, aspartic acid, and glutamic acid when the amino group is transferred to the corresponding keto acid, pyruvic, oxalacetic, or α-ketoglutaric acids. In the light of present evidence the two α-aminodicarboxylic acids appear to be the most direct link between the general metabolism of the cell and the amino acids. Glycine is an extraordinarily active substance, participating in many activities of the cell over and above its role as a protein constituent. It is a unit of several fundamentally important cell constituents, and is

TABLE 1

AMINO ACID COMPOSITION OF THE BLOOD OF CALLIPHORA AUGUR AT DIFFERENT STAGES DURING ITS LIFE-CYCLE

Amino Acid	Concentration (mM) ± Standard Error:		
	Larval Stage	Prepupal Stage	Early Pupal Stage
Alanine	13·29±0·49	4·88±0·13	3·44±0·12
Arginine	Trace	Trace	Trace
Asparagine	2·65±0·17	2·22±0·15	1·89±0·16
Aspartic acid	1·08±0·11	0·57±0·10	0·98±0·11
Cysteine and/or cystine	Trace	Trace	Trace
Glutamic acid	3·27±0·14	1·65±0·15	4·28±0·15
Glutamine	7·45±0·42	3·12±0·18	4·42±0·40
Glycine	3·93±0·10	1·82±0·11	3·25±0·10
Hydroxyproline	Trace	Trace	—
Leucine and/or isoleucine	1·51±0·06	1·46±0·06	0·84±0·06
Lysine	Trace	Trace	Trace
Methionine	Trace	Trace	Trace
Phenylalanine	1·18±0·12	1·16±0·12	1·76±0·11
Proline	8·01±0·20	1·80±0·04	5·50±0·11
Serine	2·43±0·08	1·61±0·09	2·93±0·09
Taurine	Trace	Trace	Trace
Threonine	Trace	Trace	Trace
Tyrosine	7·41±0·05	3·94±0·03	5·23±0·03
Valine	1·36±0·05	1·30±0·05	0·87±0·04

either an end-product or a precursor of substances which enter into diverse metabolic activities. The wide range of metabolic channels through which glycine is routed is given by Weinhouse (1955).

Reference to the work of Dennell (1946) on the larval cuticle of *Sarcophaga falculata* Pand. shows that in the period just prior to the prepupal stage (third to the fourth day) the cuticle undergoes a very considerable increase in thickness (from approx. 40–180μ). This increase in thickness is due to the formation of new cuticular substance and not to the absorption of water. The same is true of *C. augur* larvae. It is therefore to be expected that metabolism is at a high level throughout this period and that protein, chitin, polyphenols, and other cuticular components are being synthesized. Consequently there would be changes in the concentrations of alanine, glutamic acid, aspartic acid, glutamine, asparagine, and glycine in the two periods represented by the larval and prepupal bloods, although many other amino acids could be involved in metabolic processes. These amino acids could be incorporated directly in the cuticular proteins, and they could be taking part in other related metabolic processes. Proline could be incorporated directly in the cuticular protein, for it is known that insect cuticular proteins have a higher proline content (Hackman 1953*a*) while the overall conversion of proline into glutamic acid is well established.

Tyrosine may also be incorporated in the proteins of the cuticle for these proteins have a high tyrosine content (approx. 11·5 per cent. (Trim 1941; Hackman 1953*a*)). However, there is strong evidence to suggest that tyrosine in the blood is the precursor of the *o*-dihydric phenols which provide the quinones necessary for the tanning (i.e. hardening and darkening) of the larval cuticle to form the puparium (Dennell 1947; Fraenkel and Rudall 1947; Pryor, Russell, and Todd 1947; Hackman 1953*b*). A marked decrease in tyrosine content of the blood as the fully grown larva approaches pupation is to be expected. Dennell (1947) has brought forward evidence to show that in *S. falculata* the blood of the early pupa contains an *o*-dihydric phenol. However no phenolic compounds, other than tyrosine, could be detected in the larval, prepupal, or early pupal blood of *C. augur*.

Relatively little is known of the origin of D-glucosamine, *N*-acetyl-D-glucosamine, and chitin. From the observations of Leloir and Cardini (1953) and Lowther and Rogers (1955) it appears likely that glutamine is concerned in the biosynthesis of D-glucosamine and therefore of chitin which is built up of *N*-acetyl-D-glucosamine residues. Ussing (1946) has suggested that glutamine may function in the synthesis of uric acid in insects.

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V. REFERENCES

AUCLAIR, J. L. (1953).—Amino acids in insects. *Canad. Ent.* **85**: 63–8.

CURZON, G., and GILTROW, J. (1954).—Aromatic aldehydes as specific chromatographic colour reagents for amino-acids. *Nature* **173**: 314–5.

DALGLIESH, C. E. (1952).—The detection and differentiation of 3,4- and 2,5-dihydroxyphenyl compounds related to tyrosine. *J. Chem. Soc.* **1952**: 3943–5.

Dennell, R. (1946).—A study of an insect cuticle: the larval cuticle of *Sarcophaga falculata* Pand. (Diptera). *Proc. Roy. Soc.* B **133**: 348–73.

Dennell, R. (1947).—A study of an insect cuticle: the formation of the puparium of *Sarcophaga falculata* Pand. (Diptera). *Proc. Roy. Soc.* B **134**: 79–110.

Drilhon, A. (1952).—Étude du milieu intérieur de *Macrothylacea rubi* L. au cours de la diapause. *C.R. Acad. Sci., Paris* **234**: 1913.

Fraenkel, G., and Rudall, K. M. (1947).—The structure of insect cuticles. *Proc. Roy. Soc.* B **134**: 111–43.

Hackman, R. H. (1953*a*).—Chemistry of insect cuticle. I. The water soluble proteins. *Biochem. J.* **54**: 362–7.

Hackman, R. H. (1953*b*).—Chemistry of insect cuticle. 3. Hardening and darkening of the cuticle. *Biochem. J.* **54**: 371–7.

Hackman, R. H., and Lazarus, M. (1956).—Quantitative analysis of amino acids using paper chromatography. *Aust. J. Biol. Sci.* **9**: 281–92.

James, W. O. (1948).—Demonstration and separation of noradrenaline, adrenaline and methyladrenaline. *Nature* **161**: 851–2.

James, W. O., and Kilbey, N. (1950).—Separation of noradrenalin and adrenalin. *Nature* **166**: 67–8.

Leloir, L. F., and Cardini, C. E. (1953).—The biosynthesis of glucosamine. *Biochim. Biophys. Acta* **12**: 15–22.

Lowther, D. A., and Rogers, H. J. (1955).—Biosynthesis of hyaluronate. *Nature* **175**: 435.

Pryor, M. G. M., Russell, P. B., and Todd, A. R. (1947).—Phenolic substances concerned in hardening the insect cuticle. *Nature* **159**: 399–400.

Sarlet, H., Duchateau, G., and Florkin, M. (1952).—Les acides aminés du milieu intérieur du Ver à soie au cours du filage. *Arch. Int. Physiol.* **60**: 126–7.

Trim, A. R. (1941).—Studies in the chemistry of the insect cuticle. I. Some general observations on certain arthropod cuticles with special reference to the characterization of the proteins. *Biochem. J.* **35**: 1088–98.

Ussing, H. H. (1946).—Amino acids and related compounds in the haemolymph of *Oryctes nasicornis* and *Melolontha vulgaris*. *Acta Physiol. Scand.* **11**: 61–84.

Weinhouse, S. (1955).—"A Symposium on Amino Acid Metabolism." pp. 637–57. (Ed. W. D. McElroy and H. B. Glass.) (The Johns Hopkins Press: Baltimore.)

REGENERATION AND THE MOULTING CYCLE IN *BLATTELLA GERMANICA* L.

IV. SINGLE AND REPEATED REGENERATION AND METAMORPHOSIS

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Summary

Single or successive regenerations of metathoracic legs during the fifth instar in *Blattella germanica* L. at 29°C, 70 per cent. R.H., show the same time relations with moulting as previously described for the first instar, and do not affect the duration of development, number of moults, or other characteristics of the resulting adults.

At 70 per cent. R.H. and constant temperatures of 18, 25, or 30°C the capacity fully to regenerate a metathoracic leg persists until the onset of the last moult (metamorphosis), independent of the number of preceding moults, or regenerations of the same leg, or of age. Repeated regeneration of the same leg prolongs development and causes additional moults (supermoults), but the resulting adults are normal in size and appearance. When initiated early in development it produces up to six supermoults and prolongs duration of development by up to 300 per cent. Initiated later, it produces fewer supermoults at 25°C, whereas most animals metamorphose without supermoults at 30°C. Supermoulting has a much higher incidence in males than in females. Irregular increases in duration of the later instars, with a reduction in the amount of visible adult differentiation occurring at each moult, characterize supermoulting animals.

The indirect evidence provided by these results and other recent work suggests that, in cockroaches, the growth and differentiation hormone is quite distinct from the moulting hormone.

I. Introduction

The dependence of regeneration of the leg in *Periplaneta americana* L. on the endocrine glands has recently been demonstrated by Bodenstein (1955), who has also shown that the capacity to produce a fully differentiated regenerate persists throughout development. Other workers have reported that leg regeneration may be associated with supernumerary moults, thus involving postponement of metamorphosis, in *Blattella germanica* L. (Seamans and Woodruff 1939) and in *P. americana* (Zabinski 1936). Study of the time relations between regeneration and moulting in first instar *B. germanica* has revealed a regular interaction suggesting that regeneration both influences, and is influenced by, the endocrine mechanisms controlling the moult (O'Farrell and Stock 1953, 1954; Stock and O'Farrell 1954). The time relations between regeneration and moulting towards the end of the developmental period, the effect of regeneration on metamorphosis, and the degree of persistence of regeneration potency in face of repeated removal of the same leg each time it regenerates are all questions of interest in relation to the physiology of

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development and the mechanics of regeneration, but have so far received little attention in insects.

The present paper describes experiments designed to elucidate the following points:

(i) Do single or successive regenerations of one or both metathoracic legs in *B. germanica* follow the same general pattern, and have similar time relations with operations and ecdyses towards the end of development as they do in the first instar?

(ii) If the same metathoracic leg is removed each time it regenerates, how often can this process be repeated, and how does it affect the size and differentiation of the regenerate at various constant temperatures?

(iii) Does regeneration influence the number of moults or the total time necessary for development, or have any other effects on metamorphosis?

II. MATERIAL AND METHODS

Mass cultures and experimental animals were maintained as described previously (O'Farrell and Stock 1953). Humidity at each temperature was controlled at approximately 70±10 per cent. R.H. The work on single or successive regenerations was done at a temperature of 29±0·5°C, while the experiments on repeated regeneration were done at 18, 25, and 30°C (±1°C in each case). Experimental animals were the progeny of females from cultures kept for several generations at the experimental temperature, except at 18°C, where the parent cultures were kept at 25°C.

In the work on repeated regeneration each experimental batch was hatched from one oötheca, kept until the required age, and then subjected to removal of the left metathoracic leg at the proximal "autotomy" plane, between trochanter and femur. Thereafter, the batch was observed regularly (but not daily) at intervals appropriate to the duration of the intermoult period at the various temperatures. Regenerated legs were removed as soon as observed, and preserved, together with any animals which had died, in numbered vials. All accidentally damaged individuals were eliminated. This routine was followed until all surviving individuals in the batch were adult. The adults were preserved in separate vials according to the number of moults and regenerations they had undergone. This provided a record in material of the number of regenerations and moults, the nature of the regenerates produced at each, and the morphology of the resulting adults. Control batches were observed according to the same schedules as the corresponding experimental animals.

The routine of repeated operations was initiated at several different ages so that different numbers of repetitions could be observed at each temperature even if metamorphosis were uniform. Instars one to six were covered at 18°C, and instars two to five at 30°C. At 25°C, however, all animals underwent the first operation in the first or second instar. It was desirable to cover one of the later instars, but a shortage of animals now made it necessary to use for fourth instar coverage individuals which had already undergone operation and regeneration during their first instar. Data from these animals have been marked "†" in tabulation.

The very wide variation in timing of the later moults, even in the progeny of single inbred females, necessitated a special procedure for establishing experimental and control batches for the accurate study of time relations. Groups of egg-bearing females were isolated from the mass cultures, and a single dated subculture was established each day by pooling all progeny hatched during the preceding 24 hr. Each subculture was then observed regularly until the first fourth instar animals were seen; thereafter, daily observation was maintained to provide a supply of individuals whose age at the fourth ecdysis was known within 24 hr. Although almost 2000 animals were reared in this way during a 4-months period, it was rarely possible to obtain a substantial number of individuals entering the fifth instar not only on the same day but also at the same age. As a compromise, therefore, each experimental batch comprised individuals undergoing the fourth moult on the same day and not deviating by more than 3 days from an age of 25 days. In subdividing each such batch into experimental and control groups, care was taken to ensure that the age distribution of the 12 or fewer individuals in each group was as far as possible the same. Similar methods were used to provide a few batches of sixth and seventh instar animals of known age, but the bulk of the work was done with fifth instar material.

Removal of the left metathoracic leg between the trochanter and femur was carried out on each of the fourth, fifth, and sixth days of the fifth instar, using a total of about 400 experimental and 250 control animals. Two further series, each of about 90 animals, were subjected to successive operations, the left metathoracic leg being removed on either the fourth or the sixth day, and the right metathoracic leg on the ninth day of the fifth instar. Daily observations were continued on each batch, with appropriate recording and preservation of material, until all the animals had undergone the fifth moult. Some 200 experimental animals and 120 controls were kept under regular (but not daily) observation after the fifth moult until they were all adult.

III. Experimental Results

(a) *Single and Successive Regenerations Resulting from Operations Performed During the Fifth Instar*

Removal of one metathoracic leg during the fifth instar resulted in the appearance at the fifth ecdysis of a rounded papilla, usually containing a blastema-like mass of cells, or else of a fully differentiated hypotypical leg with tetramerous tarsus, usually about two-thirds of the length of the corresponding normal leg. Nothing resembling an intermediate stage in differentiation between a papilla and a regenerate was seen among the very few instances of abnormal regeneration observed. Where a papilla appeared, the fifth ecdysis was not delayed; where a regenerate appeared, it was delayed by a period approximating the time interval which had elapsed between the fourth ecdysis and the operation. As Table 1 shows, operations performed 3–4 days after the fourth ecdysis resulted in the appearance of regenerates at the fifth ecdysis, which was delayed by about 3 days; operations performed 4–5 and 5–6 days after the fourth ecdysis and leading to the appearance of regenerates at the fifth were responsible for an average delay in ecdysis of 4 and 5 days respectively. So far, the results corresponded remarkably closely to those described by O'Farrell and Stock (1953) for single operations in the first instar.

O'Farrell and Stock (1954) further showed that there was a "critical period" about half way through the first instar, and that regeneration "put back the clock" to establish a secondary moulting cycle with its own "secondary critical period". In the fifth instar, the work on single regenerations suggested that the critical period should occur in most animals about 5–6 days after the fourth ecdysis, and that any secondary critical period established by removing a leg earlier than 6 days after the fourth ecdysis should begin about 5 days after the operation. On these assumptions, then, successive operations separated by an interval of less than 5 days should give a preponderance of animals with two regenerates, while those separated by an interval of about 5 days should give a preponderance of animals with a regenerate on the side of the first operation and a papilla on the side of the second. As Table 2 shows, the results conformed well with this expectation. Animals which had already reached the critical period at the first operation moulted without any delay and produced two papillae; those producing a regenerate and papilla had the fifth ecdysis delayed by a period approximating to the time between the fourth ecdysis and the first operation, and those producing two regenerates had their fifth ecdysis delayed by a period approximating to the time between the fourth ecdysis and the second operation.

TABLE 1

DURATION OF FIFTH INSTAR IN RELATION TO TIME OF REMOVAL OF LEFT METATHORACIC LEG AND NATURE OF REGENERATE PRODUCED AT FIFTH ECDYSIS AT 29°C, 70% R.H.

P, rounded papilla only; R, fully differentiated regenerate with tetramerous tarsus

Interval between Fourth Ecdysis and Operation (days)	Number and Type of Regenerates Observed at Fifth Ecdysis when Occurring at Stated Number of Days after Fourth Ecdysis											
	5–6	6–7	7–8	8–9	9–10	10–11	11–12	12–13	13–14	14–15	15–16	16–17
No operation (controls)	10	29	40	47	34	41	22	15	6	4	0	0
3–4	0	0	0	0	0	23R	48R	34R	11R	0	0	0
4–5	0	2P	2P	6P	2P	1R	34R	41R	25R	2R	0	0
5–6	0	1P	3P	13P	8P	4P	0	1R	35R	49R	16R	7R

Morphologically, too, the papillae and regenerates produced after successive operations in the fifth instar were quite comparable with those arising after similar operations in the first instar. The two papillae of a pair were rather small and erratically asymmetrical in size and shape. The regenerate and papilla combination usually had large and well-differentiated regenerates, while the papillae were variable in size and blastema content. The two-regenerates combination always showed asymmetry in size and usually also in details of differentiation, the larger regenerate appearing on the side of the first operation. Only one intermediate was observed; this was on the side of the second operation.

Significant differences in the timing of the sixth and seventh ecdyses were not detected in the 183 experimental and 109 control animals followed through to metamorphosis. By analogy with the results from first instar animals, such differences would not be expected to be large, and might well be obliterated by the very wide variations in the timing of later moults; the data for the fifth moult of controls in Table 1 indicate the magnitude of these variations. Animals producing a papilla at the fifth ecdysis had a well-differentiated regenerate at the sixth, irrespective of whether metamorphosis occurred then or was postponed until the seventh ecdysis. Similarly, animals producing two papillae, or a regenerate and a papilla, at the fifth ecdysis had an almost symmetrical pair of large regenerates at the sixth, whether metamorphosis occurred or not. Those which had one or two regenerates at the fifth ecdysis showed the usual increase of symmetry at the sixth, again independently of metamorphosis.

TABLE 2

DURATION OF FIFTH INSTAR IN RELATION TO TIMES OF REMOVAL OF LEFT AND RIGHT METATHORACIC LEGS IN SUCCESSION AND NATURE OF REGENERATES PRODUCED AT FIFTH ECDYSIS AT 29°C, 70% R.H.

PP, two small, asymmetrical papillae; RP, fully differentiated regenerate on left side and papilla on right side; RI, fully differentiated regenerate on left side and an intermediate stage between papilla and regenerate on right side; RR, fully differentiated regenerates on both sides, that on the right side being smaller

Interval (days) between Fourth Ecdysis and Operation on:		Number and Nature of Regenerates Observed at Fifth Ecdysis when Occurring at Stated Number of Days after Fourth Ecdysis									
Left Side	Right Side	9–10	10–11	11–12	12–13	13–14	14–15	15–16	16–17	17–18	18–19
3–4	8–9	0	26RP	36RP	4RP	1RP	1RI	2RR	6RR	1RR	0
5–6	8–9	7PP	6PP	0	0	3RP	3RP	7RR	36RR	18RR	6RR

Finally, a few animals were observed, without attempting a detailed study of time relations, after suffering removal of the left metathoracic leg early or late in the sixth instar. Early operations yielded a well-formed regenerate at the sixth ecdysis, independently of metamorphosis. Late operations gave a typical papilla at the sixth ecdysis, which was the last for most males and some females. Those which did not metamorphose until the seventh ecdysis then produced a regenerate at that time. These results suggested that no change in the mechanism controlling regeneration in relation to moulting had taken place up to the beginning of the critical period in the penultimate instar. This conclusion was supported by the more precise work on fifth instar material, where the whole picture conformed very closely to that of first instar animals despite the fact that major changes in the direction of metamorphosis (e.g. development of gonads, elaboration of genitalia and wing rudiments) were already well under way at the time of operation. At all events, the capacity to regenerate certainly persisted up to the last possible moment before metamorphosis.

(*b*) *Influence of Repeated Regeneration on Regenerative Capacity*

In this work, the regenerated leg was always removed as soon as seen, in order to ensure the appearance of a regenerate rather than a papilla at the following ecdysis. Prolonged sequences of repetition of regeneration were observed in substantial numbers of animals at each temperature used, as shown in Table 3. Work at 18°C had to be suspended after observing a maximum of only five repetitions, because the only available cool incubator burned out and destroyed the animals. At 25°C, however, and also at 30°C, uninterrupted sequences of up to 11 repetitions were observed (associated, of course, with supernumerary ecdyses); in one instance at 25°C, a female animal produced nine regenerates in succession, failed entirely to regenerate

Table 3

SEQUENCES OF REPEATED REGENERATION OBSERVED AT THREE CONSTANT TEMPERATURES AND 70% R.H.

Temp. (°C)	Instar in which the Sequence Began	Number of Individuals Observed to Undergo Each Number of Repetitions of Regeneration Shown											
		1	2	3	4	5	6	7	8	9	10	11	12
18	First	60	59	34	19	3	—	—	—	—	—	—	—
	Second	35	27	27	22	13*	—	—	—	—	—	—	—
	Third	76	49	40	35	20	—	—	—	—	—	—	—
	Fourth	46	44	37	25*	—	—	—	—	—	—	—	—
	Fifth	42	38*	28	8*		—	—	—	—	—	—	—
	Sixth	25*	14*	5*	—	—	—	—	—	—	—	—	—
	Total, all instars	284*	231*	171*	109*	36*	—	—	—	—	—	—	—
25	First	76	42	38	28	23	21	21*	17*	15*	8*	3*	1*
	Second	18	16	15	14	14	12*	11*	3*	0	0	0	0
	Fourth	0†	23	22	22	22	21*	8*	2*	1*	0	0	0
	Total, all instars	94	81	75	64	59	54*	40*	22*	16*	8*	3*	1*
30	Second	52	50	49	48	44	41*	34*	21*	10*	4*	2*	0
	Third	35	35	34	34*	18*	8*	1*	0	0	0	0	0
	Fourth	24	22	20*	7*	1*	0	0	0	0	0	0	0
	Fifth	36	36*	18*	8*	5*	2*	0	0	0	0	0	0
	Total, all instars	147	143*	121*	97*	68*	51*	35*	21*	10*	4*	2*	0

* Total includes one or more adults. † See text, Section II.

at its tenth ecdysis, and then produced three more regenerates to make a total of 12 regenerations in 13 moults and 14 instars. About 96 per cent. of all the regenerates examined at each temperature were indistinguishable in both relative and absolute size and level of differentiation from those resulting from single operations. No serious decline in regenerative capacity with age or with frequency of repetition, therefore, appeared to be present in most of the material.

About 4 per cent. of all regenerates examined were classed arbitrarily as "defective", without attempting to distinguish degrees of defect. At 18°C, almost all the defects were minor imperfections in the tarsus, although one or two gross deformities of the whole leg occurred. The animals which had undergone seven or more moults produced about 20 per cent. of defective regenerates against the overall incidence of less than 5 per cent. Similarly, at 30°C, where the total incidence of defects was 4·3 per cent., 12 per cent. of animals undergoing eight or more moults showed defects; these included spectacular deformities such as hypertrophy, duplication of the tarsus, and one instance of total failure to regenerate. The situation at 25°C was somewhat different. Two instances of total failure to regenerate were recorded, both at the tenth ecdysis; but no gross abnormalities or even clearly-recognizable minor imperfections were observed. A rather subjective impression that the tarsi were somewhat less well differentiated in many of the animals undergoing nine or more moults remains, but it is clear that the extent of defect occurring at 25°C was substantially less than at the other two temperatures. Probably, the capacity for normal differentiation did decline somewhat, either with the approach of metamorphosis or with an increased number of repetitions of regeneration (the two factors are difficult to distinguish). This effect was not very pronounced and seems to have been almost negligible at 25°C.

Failure to regenerate was not associated with the appearance of any structure resembling a papilla; the condition rather suggested a twisting and general abortion of the trochanter. Likewise, none of the gross abnormalities observed resembled either a papilla or an intermediate stage in differentiation between papilla and normal regenerate. Among the minor defects, failure to produce a large, well-defined pretarsus with fully developed claws might perhaps be regarded as a very late "intermediate" stage in proximo-distal differentiation. On the whole, then, repeated regeneration apparently did not cause any serious suspension of the "all or nothing" principle, at least for operations performed early in any instar.

(c) *Influence of Regeneration on the Number of Moults Preceding Metamorphosis*

(i) *Effect of Single and Successive Regeneration During the Fifth Instar.*—In all, 183 experimental and 109 control animals were followed through to metamorphosis at 29°C from the time relations experiments on fifth instar material. Table 4 shows that there was no marked difference between regenerating animals and controls in the number of moults preceding metamorphosis. The proportion of control females undergoing the extra (seventh) moult in different batches varied between 25 and 75 per cent. Hence no significance was attached to the differences in this respect between regenerating animals undergoing different treatments (Table 4). The rather high proportion of males undergoing the seventh moult after producing a papilla at the fifth and a regenerate at the sixth ecdysis may have been significant, since very few control or other males had this extra moult. Two females also had an eighth moult after producing two regenerates at the fifth ecdysis; these moults were not paralleled in any of the other animals, and were therefore true supernumerary moults.

In none of the experimental animals of Table 4 was the age at metamorphosis outside the range of variation observed in the controls (viz. 40–52 days in animals undergoing six moults and 50–65 days in animals undergoing seven moults). The last moult of experimental animals tended to begin a little later than in controls, presumably because of the still uncompensated delay in the fifth moult caused by regeneration, but a significant difference between experimental animals and controls in average age at metamorphosis was not demonstrated. It was therefore concluded that, apart from the obviously exceptional individuals mentioned above, neither the number of moults nor the duration of development was influenced by regeneration during the fifth instar.

TABLE 4

EFFECT OF REMOVAL OF ONE OR BOTH METATHORACIC LEGS DURING THE FIFTH INSTAR ON THE NUMBER OF MOULTS PRECEDING METAMORPHOSIS AT 29°C, 70% R.H.

Regenerates Produced at the Fifth Ecdysis	Number and Sex of Individuals Metamorphosing at:								
	Sixth Ecdysis			Seventh Ecdysis			Eighth Ecdysis		
	♂	♀	Total	♂	♀	Total	♂	♀	Total
None (controls)	46	16	62	3	44	47	0	0	0
One papilla	2	3	5	5	5	10	0	0	0
One regenerate	50	33	83	5	20	25	0	0	0
Two regenerates	25	9	34	1	25	26	0	2	2

(ii) *Effect of Repeated Regeneration.*—At all the experimental temperatures, the great majority of control males metamorphosed at the sixth ecdysis. The proportion of control females undergoing an extra (seventh) moult again varied between 25 and 75 per cent. in different batches. As regenerates can appear only at an ecdysis, it will be clear from Table 3 that many of the experimental animals had more than seven moults (the maximum observed was, in fact, 13). The seventh moult, being absent in almost all control males and many control females, is effectively a supernumerary moult, but it has nothing to do with regeneration. Additional moults associated with regeneration, beginning with the seventh in males and the eighth in females, will therefore be termed "supermoults" rather than "supernumerary moults" in this paper (see Table 6). Tables 5 and 6 taken together provide a summary of the data obtained on the relationship between repeated regeneration and supermoulting.

TABLE 5

SURVIVAL AND METAMORPHOSIS OF ANIMALS UNDERGOING VARIOUS SEQUENCES OF REPEATED REGENERATION AND SIX OR MORE ECDYSES AT THREE CONSTANT TEMPERATURES AND 70% R.H.

Temp. (°C)	Instar in which the Sequence Began	Number of Adults and Non-Adults Undergoing Number of Ecdyses Shown:														
		6		7		8		9		10		11		12		13
		Adult	Non-Adult	Adult	Non-Adult	Adult	Non-Adult	Adult	Non-Adult	Adult	Non-Adult	Adult	Non-Adult	Adult	Non-Adult	Adult only
18	First	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Second	3	10	0	0	0	0	0	0	0	0	0	0	0	0	0
	Third	0	35	0	20	0	0	0	0	0	0	0	0	0	0	0
	Fourth	0	37	1	24	0	0	0	0	0	0	0	0	0	0	0
	Fifth	1	37	0	28	1	7	0	0	0	0	0	0	0	0	0
	Sixth	2	23	6	8	1	4	0	0	0	0	0	0	0	0	0
	Total, all instars	6	142	7	80	2	11	0	0	0	0	0	0	0	0	0

TABLE 5 (*Continued*)

Temp. (°C)	Instar in which the Sequence Began	Number of Adults and Non-Adults Undergoing Number of Ecdyses Shown:														
		6		7		8		9		10		11		12		13
		Adult	Non-Adult	Adult	Non-Adult	Adult	Non-Adult	Adult	Non-Adult	Adult	Non-Adult	Adult	Non-Adult	Adult	Non-Adult	Adult only
25	First	0	21	3	18	2	15	6	9	3	5	2	1*	0	1*	1*
	Second	0	14	1	11	8	3	3	0	0	0	0	0	0	0	0
	Fourth	0†	22	0	22	13	8	6	2	1	1	1	0	0	0	0
	Total, all instars	0	57	4	51	23	26	15	11	4	6	3	1	0	1	1
30	Second	0	44	3	38	10	24	6	15	5	5	2	2	2‡	0	0
	Third	16	18	10	8	7	1	1	0	0	0	0	0	0	0	0
	Fourth	12	8	6	1	1	0	0	0	0	0	0	0	0	0	0
	Fifth	18	18	8	10	1	7	2	3	1	1	1?§	0	0	0	0
	Total, all instars	46	88	27	57	19	32	9	18	6	6	3	2	2	0	0

* Failed to regenerate at the tenth ecdysis only.

† See text, Section II.

‡ "Nymphoid" female included (see text, Section III (*e*)).

§ Died in ecdysis, too shrivelled to dissect, but certainly male and probably metamorphosing.

Table 5 shows the number of animals, including non-adults, surviving at each ecdysis and temperature. Only 11 males and 4 females metamorphosed at 18°C before the breakdown of the incubator. Eight males supermoulted but no females did so, 80 eighth instar and 11 ninth instar non-adults were observed at 18°C, and some of these would have undergone at least three supermoults had they lived to metamorphose. At 25 and 30°C numerous adults were reared and the data on non-adults given in Table 5 are of less interest in relation to supermoulting; they do, however, show that mortality was much lower at the higher temperatures.

TABLE 6

NUMBER OF SUPERMOULTS ASSOCIATED WITH REPEATED REGENERATION AT 25 AND 30°C, 70% R.H.

The first supermoult is assumed to be the seventh moult for males and the eighth for females.

Temp. (°C)	Instar in which the Sequence Began	Number and Sex of Animals Metamorphosing After the Number of Supermoults Shown:														Total, All Ecdyses
		0		1		2		3		4		5		6		
		♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	
25	First	0	1	2	0	2	5	1	2	1	2	0	0	0	1*	17
	Second	0	1	0	5	3	1	2	0	0	0	0	0	0	0	12
	Fourth†	0	0	0	5	8	4	2	1	0	1	0	0	0	0	21
	Total, all instars	0	2	2	10	13	10	5	3	1	3	0	0	0	1*	50
30	Second	0	0	3	6	4	3	3	3	2	2	0	1‡	1	0	28
	Third	11	10	5	3	4	1	0	0	0	0	0	0	0	0	34
	Fourth	6	8	4	1	0	0	0	0	0	0	0	0	0	0	19
	Fifth	9	14	3	0	1	1	1	0	1	0	1?§	0	0	0	31
	Total, all instars	26	32	15	10	9	5	4	3	3	2	1?§	1	1	0	112

* Failed to regenerate at the tenth ecdysis only.

† See text, Section II.

‡ "Nymphoid" female included (see text, Section III (*e*)).

§ Died in ecdysis, too shrivelled to dissect, but certainly male and probably metamorphosing.

The data on supermoulting of animals reaching metamorphosis, presented in Table 6, were derived from 21 males and 29 females reared at 25°C and from 59 males and 53 females reared at 30°C. All but two (both females) had at least one supermoult at 25°C, but at 30°C 44 per cent. of males and 60 per cent. of females metamorphosed without a supermoult. The sequence initiated in the second instar at 30°C, however, yielded supermoults in all the 13 males and 15 females reared: the general pattern in this group broadly resembled that of the 6 males and 11 females reared at

25°C from the sequence initiated in the first instar, with many animals undergoing three or more supermoults, up to a maximum of six. At 25°C, sequences initiated in the second and fourth instars had a lower incidence of supermoults than that initiated in the first instar, and the maximum number recorded was only four (one case). In these sequences all males had at least two supermoults, but females with two or more were limited to one in the second instar sequence, and to six (55 per cent. of the number of females reared) with the fourth instar sequence. The latter figure may be influenced by the fact that the animals had already regenerated in the first instar before the sequence was started in the fourth (see Section II). With the first instar sequence, however, the incidence of two or more supermoults was relatively higher in females than in males, in the ratio of about 7:5. The otherwise similar second instar sequence at 30°C had this ratio reversed (4:3 in favour of males). In other sequences at 30°C the preponderance of supermoults in males was very marked. About 15–16 per cent. of the adults of both sexes reared at 30°C from the third and fifth instar sequences had two or more supermoults, but none of those from the fourth instar sequence had more than one. Thus it appeared that the effect of repeated regeneration on the number of moults may have fallen to a minimum for sequences initiated about the middle of the developmental period (i.e. the fourth instar). The same principle appeared to operate to some extent at 25°C, but comparable sequences began one moult earlier than at 30°C. Without further data this interpretation can only be regarded as provisional. But the following definite conclusions can be drawn from the data:

(1) Repeated regeneration is associated with supermoults at 18, 25, and 30°C. The incidence of supermoults is greater at 25 than at 30°C.

(2) Sequences of repeated regeneration initiated in the first instar at 25°C and in the second instar at 30°C produce a greater incidence of supermoults than those begun in later instars.

(3) The number of supermoults undergone by individuals is similar at 25 and 30°C, rarely exceeding three and exceptionally exceeding four.

(4) Except in sequences begun in the first instar at 25°C, males tend to undergo supermoults more readily than females, and do so much more frequently in sequences begun in the third, fourth, or fifth instars at 30°C.

(*d*) *Influence of Regeneration on Duration of Postembryonic Development*

The duration of development was normal in animals regenerating only in the fifth instar at 29°C (see Section III (*c*) (i)). For those undergoing repeated regeneration, no satisfactory information was obtained at 18°C, but the data for 25 and 30°C are presented in Table 7. The age of metamorphosis became more and more variable with an increased number of moults, and wide overlaps occurred. Table 7 therefore shows the range of maximum and minimum ages observed, as well as the average age at metamorphosis after each number of ecdyses. The table takes no account of the instar in which the sequence was initiated nor of the sex of the animals, because no consistent differences were recognized in relation to these points.

Evidently the duration of development was hardly increased in animals metamorphosing at the sixth or seventh ecdysis, although at 30°C the average duration at the seventh ecdysis of experimental animals approximated to the maximum duration recorded for controls. As the number of ecdyses increased beyond seven, the duration of development became more and more prolonged, reaching about three times the control average in the slowest animal at 25°C, and four times the control average in two animals at 30°C. This prolongation resulted not only from the increased number of instars but also from an increase in their average duration. In controls, the average duration of the first instar was about 10 days at 25°C and 5 days at 30°C. The mean duration of an instar over the whole developmental period, however, was about 13 days at 25°C and 7 days at 30°C. This was due to a general slowing down in the rhythm of moulting, such that one or more of the later instars approached or exceeded twice the duration of the first. A similar process apparently continued with the supermoults of regenerating animals, so that instars with a duration of the order of 30 days occurred in some supermoulting animals at 30°C.

TABLE 7

AGE AT METAMORPHOSIS AFTER VARIOUS NUMBERS OF MOULTS IN ANIMALS UNDERGOING REPEATED REGENERATION AT 25 AND 30°C, 70% R.H.

Ages are given in days from hatching, correct to the nearest 3 days. For controls at 25°C, the range was 66–105 days, average 84 days. For controls at 30°C, the range was 32–56 days, average 42 days. Controls undergoing seven ecdyses tended towards the upper figure at each temperature. N.R., not recorded

Temp. (°C)	Range and Average of Ages (days) Recorded at Metamorphosis at Each Ecdysis Shown:													
	Sixth		Seventh		Eighth		Ninth		Tenth		Eleventh		Twelfth		Thirteenth
	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range
25	N.R.	—	74–98	92	94–143	112	119–185	150	144–175	160	186–203	196	N.R.	—	247*
30	39-54	44	46-75	56	56–100	70	69–102	88	88–137	98	117–134	122	168–190	180	N.R.

* One animal only.

It unfortunately proved impossible to devise a satisfactory method of following through the development of individual animals, except where they had some peculiarity of facies or were the sole survivors of an experimental batch. The five slowest growing animals at 30°C (not all in one batch) had a peculiar facies which made them recognizable from the seventh moult onwards (see Section III (*e*)). At the seventh ecdysis their ages ranged from 75 to 97 days, and the four which survived metamorphosed at the eleventh and twelfth ecdyses, being the only animals to attain such a number. This suggested the possibility that some animals might be "pre-destined" to a large number of supermoults by slow growth at quite an early stage. Table 8 was therefore constructed from the data of the second instar sequence at 30°C, the only one with sufficient numbers undergoing several supermoults to permit this sort of analysis. Small numbers and wide variations in the later moults made analysis beyond the ninth ecdysis pointless.

Clearly there was a sharp bimodal distribution in the ages of non-adult animals at the sixth to ninth ecdyses. Animals of group A completed a given moult several days before any of group B began it. Metamorphosis at a given ecdysis conformed to group A timing rather than group B. Nevertheless, groups A and B did not comprise the same individuals from one moult to the next. At the ninth ecdysis, 16 adults and group A non-adults were recorded, yet only 11 group A non-adults were present at the eighth ecdysis. Thus the group B of the eighth ecdysis must have contributed some of the adults or group A non-adults recorded at the ninth. On mean ages this seems difficult, but the earliest-moulting group B individuals of the eighth ecdysis could have produced the latest-moulting group A or adult individuals of the ninth ecdysis, given a rather short intermoult period of the order of 12–14 days. Thus intermoult periods did not undergo a regular increase with the number of ecdyses; animals undergoing large numbers of supermoults had some exceptionally long intermoult periods (classifying them in group B) followed by a restoration of normal periods one or more moults before metamorphosis. In the abnormal animals with 12 moults, however, restoration of a more normal moulting rhythm at the ninth or tenth ecdysis was followed by a very long intermoult period immediately before metamorphosis (30 days for the male, 28 days for the female). Thus repeated regeneration in the animals of Table 8 seems to have been associated with a rather irregular fluctuation rather than a consistent progressive increase in the duration of intermoult periods. The occurrence of at least one very long intermoult period in supermoulting animals seems to be established, however, although it can not be precisely related to the physiological onset of metamorphosis.

TABLE 8

AGES AT SIXTH TO NINTH ECDYSES OF ANIMALS UNDERGOING REPEATED REGENERATION SEQUENCE INITIATED IN SECOND INSTAR AT 30°C, 70% R.H.

Means are given in days from hatching, standard errors to the nearest day

Animal Groups	Age Statistics (days) for:							
	Sixth Ecdysis		Seventh Ecdysis		Eighth Ecdysis		Ninth Ecdysis	
	No. of Animals	Mean ± S.E.	No. of Animals	Mean ± S.E.	No. of Animals	Mean ± S.E.	No. of Animals	Mean ± S.E.
Non-adults, group A	30	44 ± 1	22	56 ± 1	11	66 ± 2	10	87 ± 4
Non-adults, group B	14	56 ± 1	14	75 ± 3	13	91 ± 5	5	120 ± 4
Adult males	0	—	3	55 ± 3	3	67 ± 3	3	87 ± 12
Adult females	0	—	0	—	6	65 ± 3	3	92 ± 7

(e) *General Effects of Regeneration*

Both adults and non-adults appeared to be morphologically and functionally normal after undergoing single or successive regenerations in the fifth instar at 29°C.

In the animals undergoing repeated regeneration, supermoulting was not accompanied by gross morphological peculiarities in the resulting adults, which were distinguishable from controls only by the presence of the regenerate. "Super-differentiation", like that described by Bodenstein (1953) for *Periplaneta* adults was not observed in any of the experimental animals; these, of course, had undergone their supermoults before and not after metamorphosis. At 18°C, the few adults reared showed several instances of failure of the wings to expand normally, both in controls and experimental animals; this effect was not observed at higher temperatures and seems unlikely to be related to regeneration.

The only striking abnormality observed was that of the single female undergoing 12 moults at 30°C. This was very small, with imperfectly developed wings and genitalia, but a predominantly adult appearance, somewhat recalling the "nymphoids" described by Scharrer (1946) in *Leucophaea*. It died 15 days after the twelfth ecdysis without showing any sign of further moulting. The ovaries were somewhat deformed, containing white crystalline deposits which were also unusually abundant in the fat-body and even occurred in the coxal musculature of the regenerated leg. The internal organs otherwise appeared normal.

Non-adult animals destined to undergo several supermoults were recognizable at the sixth or seventh ecdysis; they were obviously abnormally small and had less differentiation of the wing buds and external genitalia than normal. An animal destined for 10 moults did not present at its seventh ecdysis the appearance of a normal fourth instar animal, but of something intermediate in size and characteristics between the normal fourth instar and the normal pre-adult. The appearance in supermoulting animals of the morphological changes leading towards metamorphosis was, therefore, not delayed in its onset, but merely retarded by being spread over an increased number of moults. Animals undergoing more than 10 moults at 30°C, however, were grossly abnormal in appearance from at least the seventh ecdysis. They were very small, although not resembling the pathological first instar "dwarfs" observed by O'Farrell and Stock (1953). The cuticle was pale, nut-brown rather than the shining black or dark brown of normal non-adults, and the pale appearance was accentuated by unusually heavy white crystalline deposits in the fat-body, visible through the translucent cuticle. Some other experimental animals and a few controls, especially at 30°C, showed similar but less marked peculiarities. These were associated with lowered viability, an increased number of moults, and some deformity of the gonads, especially in females. Apparently these peculiarities resulted from some sporadically occurring metabolic disturbance, possibly related to the intracellular symbionts (Brooks and Richards 1955).

IV. Discussion

Regeneration may sometimes be a local or largely localized phenomenon, as in the crustacean *Asellus* (Needham 1949); there is in this animal a decline and recovery of regeneration potency extending over a series of repetitions of regeneration of the same appendage. In *B. germanica*, however, the metathoracic leg retains its regeneration potency almost unimpaired over a series of repeated regenerations far exceeding the normal number of moults. This continues up to the physiological onset of metamorphosis, which is often delayed by a number of supermoults, suggesting that regeneration of the metathoracic leg in *B. germanica* is a systemic rather than a local phenomenon. Such decline in regeneration potency as is observed is qualitative (e.g. imperfectly formed pretarsi) rather than quantitative (e.g. reduction in size of regenerates). It is also possible that the adult expectation of life may be influenced by the slowing down of development associated with repeated regeneration, but this has not been investigated.

The present work shows that the overall course of events in regeneration and moulting is the same in the fifth instar as in the first. It is, therefore, unnecessary to postulate a special interaction between repeated regeneration and adult differentiation; the results should be explicable in terms of the relationships already demonstrated between regeneration and the moulting cycle (O'Farrell and Stock 1953, 1954; Stock and O'Farrell 1954). The effects of repeated regeneration on metamorphosis can be interpreted as an extension of the already postulated disturbance of endocrine balance. The virtual absence of nymphoids, of gigantism, and of super-differentiation in animals, whose adult differentiation was spread over a greatly increased number of moults, suggests that competition between the regenerate and the organism as a whole for the differentiation promoting hormone(s) is a self-regulating mechanism (compare the "compensation" of delay in moulting due to a single regeneration discussed by O'Farrell and Stock 1953). The hormone balance required for development to proceed normally may be most critical in the early instars, since it is then that the initiation of repeated regeneration has the greatest effect in prolonging development and inducing supermoults, presumably through the draining off of differentiation hormone(s) by the regenerate. Maturation of the testes in the male, however, may make additional demands on the hormone(s) concerned during the middle of the larval period; this may explain the higher incidence of supermoulting seen in males in which repeated regeneration was initiated comparatively late in development. Given a shortage of hormone(s), the prolongation of one or more intermoult periods may be explained by the necessity for restoring concentrations to a level sufficient to permit moulting.

Novak (1951*a*, 1951*b*) regards metamorphosis as the outcome of balanced competition between adult differentiation, due to an intracellular factor in the tissues, and larval differentiation under the influence of the juvenile hormone of the corpus allatum in the presence of a moulting hormone which is not responsible for promoting growth and differentiation. On this hypothesis repeated regeneration might be expected to produce additional moults; but these would occur, if anything, in a shortened developmental period, and at least some of the resulting adults should be abnormal. The overall time relations between single and successive regenerations and moulting are also difficult to explain on Novak's theory.

The majority of workers (see Wigglesworth 1954) consider that metamorphosis results from changes in the balance between two antagonistic hormone systems, one a "juvenile" hormone and the other promoting both moulting and growth (including adult differentiation). This theory can explain the observed time relations between single and successive regenerations and moulting, as well as the absence of premature metamorphosis and aberrant adults in repeatedly regenerating animals, by reference to the idea of a balanced system. If, however, the postponement of metamorphosis in repeatedly regenerating animals is due to a deficiency in a growth and differentiation hormone also responsible for moulting, the observed results are difficult to interpret: numerous supermoults occur during the very period when the hormone deficiency is preventing metamorphosis. This difficulty is resolved by assuming that (at least in *B. germanica*) two linked but distinct hormones are responsible for moulting on the one hand and for growth and adult differentiation on the other. Utilization of the growth hormone in regeneration might abolish the competence of the tissues to respond to the moulting hormone until a normal balance was restored, as suggested by O'Farrell and Stock (1953) in explaining the time relations of single regeneration and moulting. With repeated regeneration, a moult would then occur each time the level of growth hormone became sufficient to restore tissue competence towards the moulting hormone; this level of growth hormone would not necessarily be high enough to bring about metamorphosis.

The difficulty of identifying by direct methods the many elements probably present in the hormone complex of insects seems to have led to caution in suggesting additions to their number. The indirect evidence provided by the present work seems to justify the suggestion that in *B. germanica* there may be a moulting hormone entirely distinct from, but functionally connected with, the growth and differentiation hormone.

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VI. References

Bodenstein, D. (1953).—Studies on the humoral mechanisms in growth and metamorphosis of the cockroach, *Periplaneta americana*. I. Transplantation of integumental structures and experimental parabiosis. *J. Exp. Zool.* **123**: 189–232.

Bodenstein, D. (1955).—Contributions to the problem of regeneration in insects. *J. Exp. Zool.* **129**: 209–24.

Brooks, M. A., and Richards, A. G. (1955).—Intracellular symbiosis in cockroaches. I. Production of aposymbiotic cockroaches. *Biol. Bull.* **109**: 22–39.

Needham, A. E. (1949).—Depletion and recuperation of the local factors during repeated regeneration. *J. Exp. Zool.* **112**: 207–32.

Novak, V. J. A. (1951*a*).—New aspects of the metamorphosis of insects. *Nature* **167**: 132–3.

Novak, V. J. A. (1951*b*).—The metamorphosis hormones and morphogenesis in *Oncopeltus fasciatus* Dal. *Mém. Soc. Zool. Tchécosl.* **15**: 1–48.

O'Farrell, A. F., and Stock, A. (1953).—Regeneration and the moulting cycle in *Blattella germanica* L. I. Single regeneration initiated during the first instar. *Aust. J. Biol. Sci.* **6**: 485–500.

O'Farrell, A. F., and Stock, A. (1954).—Regeneration and the moulting cycle in *Blattella germanica* L. III. Successive regeneration of both metathoracic legs. *Aust. J. Biol. Sci.* **7**: 525–36.

Scharrer, B. (1946).—The role of the corpora allata in the development of *Leucophaea maderae* (Orthoptera). *Endocrinology* **38**: 35–45.

Seamans, L., and Woodruff, L. C. (1939).—Some factors influencing the number of molts of the German roach. *J. Kans. Ent. Soc.* **12**: 73–6.

Stock, A., and O'Farrell, A. F. (1954).—Regeneration and the moulting cycle in *Blattella germanica* L. II. Simultaneous regeneration of both metathoracic legs. *Aust. J. Biol. Sci.* **7**: 302–7.

Wigglesworth, V. B. (1954).—"The Physiology of Insect Metamorphosis." 152 pp. (Cambridge Monographs in Experimental Biology No. 1). (Cambridge Univ. Press.)

Zabinski, J. (1936).—Inconstancy of the number of moults during the post-embryonic development of certain Blattidae. *Ann. Mus. Zool. Polon.* **11** (12).

THE PRE-NATAL DEVELOPMENT OF WOOL FOLLICLES IN MERINO SHEEP

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Summary

A histological study has been made of wool follicles in skin from the trunk of 24 sheep foetuses ranging in age from 69 to 145 days of gestation. Fifteen of these foetuses are known to be entirely Merino in origin, and the remainder are believed to be wholly or predominantly Merino. Extensive use has been made of serial sections parallel to the axis of the wool follicles as well as those parallel to the skin surface. Skin from Merino lambs and adults has also been examined.

A series of stages in the development of hair follicles in mammals is defined. The special features of each stage in the development of primary and secondary wool follicles in the Merino are indicated. Secondary follicle development, not fully described hitherto for any breed of sheep, shows some distinctive features, the most important of which is the origin of later follicles by branching from earlier ones. Thus there are two types of secondary follicles, original and derived. Some preliminary information has been obtained about their development and structure in late pre-natal and early post-natal life.

Carter's (1943) proposed series of stages of wool follicle group development, based on the appearance of transverse sections of skin at the level of the sebaceous glands, are re-defined in the light of the present observations and accompanied by a table showing the stage of development of each type of follicle.

Attention is drawn to the need for further study of the way in which branching follicles contribute to the development of the wool fibre population in Merinos and other breeds of sheep. Some possible implications for current researches on this subject are discussed.

I. Introduction

At the present time there is widespread interest in the study of the development of the wool follicle population in sheep, and an awareness of its importance for the understanding of the adult fleece and the control of wool production. This is evident from the number of recent papers on post-natal wool follicle development, including those by Burns (1953, 1954*a*, 1954*b*), Fraser (1952*a*, 1952*b*, 1953, 1954), Fraser and Short (1952), Margolena (1954), Schinckel (1953, 1955*a*, 1955*b*), and Short (1955*a*, 1955*b*). Various features in the development of wool follicles during the pre-natal period have been described by Spöttel and Tänzer (1923), Duerden and Ritchie (1924), Tänzer (1926), Wildman (1932), Carter (1943), and Carter and Hardy (1947). Yet, when the present authors commenced a study of the development of foetal sheep skin and wool in tissue culture (Hardy and Lyne 1956*b*), they found that none of the published accounts was adequate for a comparison between the development of wool follicles *in vivo* and *in vitro*. The time seemed opportune for a more precise account of pre-natal follicle development in the Merino, including some processes which have

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hitherto received little attention. It is the aim of this paper to give such an account. A preliminary note concerning this work has already been published (Hardy and Lyne 1956*a*).

II. Material and Methods

(*a*) *Animals*

Twenty-four sheep foetuses from a number of sources provided material for this study. While 15 of these were known to be entirely Merino in origin, it was not possible to check on the alleged Merino origin of the nine others. However, the foetuses in the latter group were either wholly or predominantly Merino, and were similar in external features, body dimensions, and skin structure to those of known parentage of the same age, so they were included in the observations.

The G series of 12 South African Merino foetuses of known ages ranging from 69 to 145 days had already been used for wool follicle development studies by Carter (1943) and Carter and Hardy (1947), and is described in the latter paper. The KR series of seven undated foetuses believed to be wholly or predominantly Merino in origin, and foetus No. X401, a Merino foetus, were sampled by one of us (M.H.H.) and the skin sections were used in a previous study (Carter and Hardy 1947). The S series of four foetuses was used also for tissue culture experiments (Hardy and Lyne 1956*b*). Two undated foetuses were believed to be Merinos. The other two, 98 and 125 days old, were of fine-woolled Merino (non-Peppin) parentage.

A further estimate of the age of all dated foetuses from their body dimensions was made by a modification of Cloete's (1939) technique as described by Carter and Hardy (1947), and good agreement was obtained between estimated and true ages. The external features were compared with those described by Cloete as an additional check. Estimated ages obtained by the same means were used in all calculations for the undated foetuses.

In addition to the pre-natal material, skin samples from Merinos have been studied from birth to 7 days of age, at 2, 4, 5, 6, 18, and 19 weeks, and at 9 and 18 months.

(*b*) *Histological Methods*

Entire foetuses and most of the skin samples were fixed in formalin, but a few skin samples were fixed in Zenker's fluid. The sampling positions, all from the fleece-bearing area, were chosen according to the system described by Carter and Hardy (1947), and included a mid-side sample for every foetus. Serial sections at 7 or 8 μ, or occasionally thicker, were cut transversely through the wool follicles and, when possible, in the longitudinal direction as well. They were stained with haemalum and eosin, and, in all except the G series, picric acid was added to stain the wool fibres.

Many individual follicles and follicle groups were studied in complete serial sections in either the longitudinal or the transverse direction, and reconstructions were made from camera lucida drawings.

(*c*) *Terminology*

Reference will be made to primary follicles (P) and secondary follicles (S) as defined by Wildman and Carter (1939), and to follicle groups as defined by Carter

(1943). Types of secondary follicles will be defined in this paper. The following additional symbols for follicle types recently proposed by Hardy and Lyne (1956*c*) will be used: *PC* = central *P* of trio group; *PL* = lateral *P* of trio group; *PCX* = member of first-formed *PC*; *PCY* = member of later-formed *PC*; *PLx* = *PL* associated with *PCX*; *PLy* = *PL* associated with *PCY*; *SO* = original *S*; *SD* = derived *S*; *SOU* = unbranching *SO*; *SOB* = branching *SO*. The symbol *f* (follicle containing a fibre) is added where necessary to distinguish follicles which have reached this stage of development.

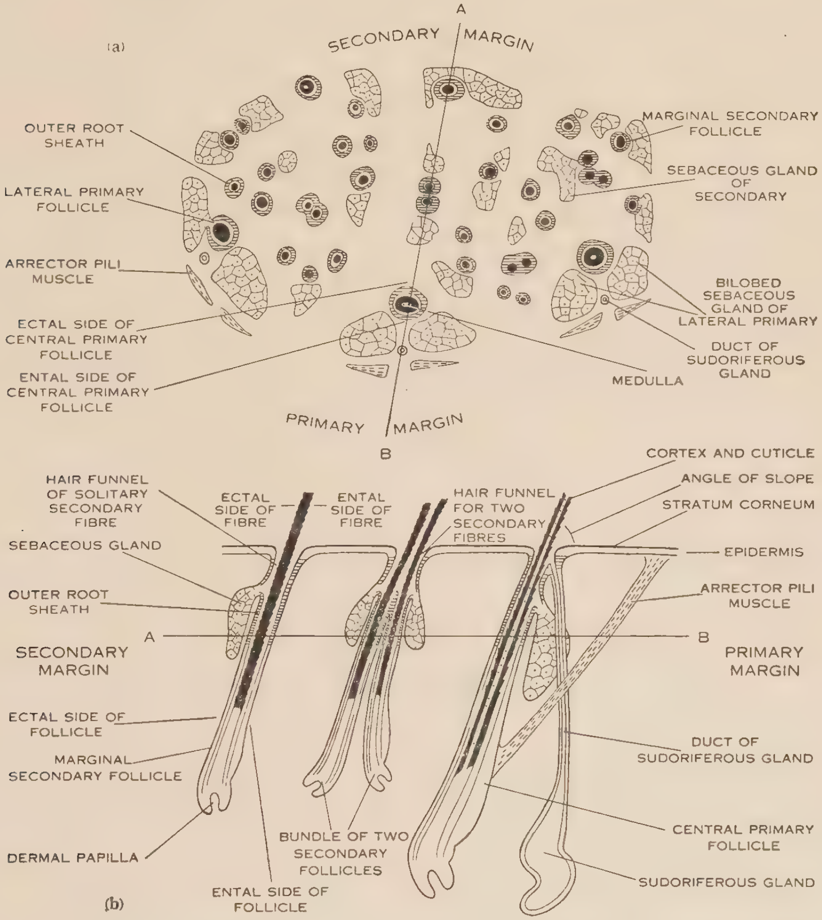


Fig. 1.—(*a*) Diagrammatic transverse section through an adult Merino wool follicle group at the level of the sebaceous glands. (*b*) Diagrammatic longitudinal section through the follicles and accessory structures in or near the plane passing through A–B shown in Figure 1 (*a*).

The terms for parts of the follicle group used by Carter (1943) and additional terms used in this paper are illustrated in Figure 1. The *angle of slope* of an emergent fibre (or a follicle), is the acute angle which the axis of the fibre (or follicle) makes with its projection on the skin surface. Auber (1950) and earlier authors refer to the *ental* side of an emergent *fibre* as that on the side of the acute angle of slope and the *ectal* side as the opposite one. Thus the *ental* side of a *follicle* is that which makes an obtuse angle with the skin surface and the *ectal* side is the one opposite. The *neck* of a developing or mature follicle is the epidermal part from the opening of the sebaceous gland to the base of the epidermis. The term *funnel* (Duerden and Ritchie 1924) indicates the cavity between the wool fibre and the follicle wall which extends from about the level of the opening of the sebaceous gland to the surface of the skin. A *follicle bundle* is an aggregation of two or more follicles which are joined over part of their length, whether by origin from a common epidermal downgrowth or by fusion of originally separate follicles.

III. The Development of Individual Follicles

The sequence of development of a mammalian hair follicle may conveniently be described by a series of stages. The following definitions of follicle stages are based on those proposed by Hardy (1949) for the mouse but are widened to make them applicable to other rodents which have been examined, to sheep, and possibly to a variety of mammals. Each stage number is prefixed by the letter *F* to distinguish it from the stages of follicle groups to be described later.

Stage F1: *Follicle plug.*—A plug of cells from the epidermis (or primitive ectoderm) extends into the dermis (or primitive mesoderm). This stage begins when a localized thickening of the basal layer of the epidermis can be recognized. There is frequently an aggregation of dermal cells beneath the epidermal plug.

Stage F2: *Pre-papilla.*—The base of the epidermal plug flattens prior to invagination.

Stage F3: *Papilla.*—The base of the epidermal plug is invaginated and a dermal papilla is formed. This stage begins when the base of the epidermal plug becomes recognizably concave.

Stage F4: *Hair cone.*—The elongated cells of Henle's layer of the inner root sheath are recognized, forming a cone which is directed along the axis of the follicle towards the skin surface.

Stage F5: *Advanced hair cone.*—The tip of the hair cone reaches the deepest level of the sebaceous gland, if present, or the deepest level of the sebaceous glands of adjacent follicles of the same type.

Stage F6: *Hair formation.*—The tip of a keratinized hair fibre appears inside the hair cone.

Stage F7: *Hair in epidermis.*—The tip of the keratinized hair reaches beyond the base of the epidermis above the follicle. A hair canal has usually been formed in the epidermis before the hair tip reaches this level.

Stage F8: *Hair emerged.*—The tip of the hair has penetrated the superficial layers of the epidermis.

The above stages can be recognized in both the primary and secondary follicles of sheep. The special features in the development of each type of follicle in the Merino will be considered in the next section.

The development of hair canals in association with wool follicles in sheep will be described in detail in a subsequent paper. Briefly, the hair canal is formed by two separate processes—keratinization of cells in the epidermis and disintegration of sebaceous cells which have migrated to the neck of the follicle. In the epidermal cells there is keratohyalin granulation and keratinization which is followed by the appearance of an intercellular space, while in the sebaceous cells there is cell degeneration followed by space formation. Eventually a continuous canal runs from the follicle neck to the upper part of the epidermis. The development of hair canals does not always take place during identical stages of follicle development, but in the following descriptions reference will be made to the average state of the hair canal at each follicle stage.

(*a*) *Primary Follicles*

Several authors have made observations on the development of primary follicles in sheep. Spöttel and Tänzer (1923) studied a few foetuses of the Merino and two other breeds. The descriptions of follicle development in the South African Merino by Duerden and Ritchie (1924) and in British breeds by Wildman (1932) clearly referred only to primary follicles, although the authors do not emphasize this point. Whilst Duerden and Ritchie and also Wildman studied follicles mainly in longitudinal sections, Carter (1943) based his description of primary follicles in the Australian Merino entirely on transverse sections.

A more complete picture of follicle development is made possible by the use of both longitudinal and transverse sections. Since most of the previously published observations were confirmed in the present study, the following account will be limited to the special features of *P* follicles in the Merino at each of the defined mammalian stages (Fig. 2). This description applies to both the central *P* (*PC*) and lateral *P* (*PL*) follicles, which were studied separately.

Stage F1.—At the beginning of this stage an aggregation of dermal cells appears beneath the epidermal plug, which usually has an acute angle of slope.

Stage F2.—The base of the epidermal plug flattens when it is still relatively short, the length of the plug being usually not more than twice its diameter. Near the beginning of this stage the sudoriferous gland rudiment appears as a solid bud from the ental side of the follicle (stage *F2a*). Later the bilobed sebaceous gland bud is formed on the ental side below the sudoriferous gland rudiment, and differentiated sebaceous cells can be recognized (stage *F2b*). These observations agree with those of Wildman (1932) and Carter (1943), and are contrary to those of Duerden and Ritchie (1924), who stated that in the Merino foetus the sudoriferous gland rudiment appeared later than that of the sebaceous gland.

Stage F3.—This stage can be subdivided according to the shape of the dermal papilla. At stage *F3a* the length of the papilla is less than its diameter, and at stage *F3b* its length is equal to or (rarely) greater than the diameter. Sometimes the solid sudoriferous gland rudiment shows a distal swelling, and the first trace of the arrector pili muscle may be found. Formation of the epidermal part of the hair canal may begin at *F3a*, and sebaceous cells have sometimes migrated to the follicle neck at *F3b*.

Stage F4.—This stage is more difficult to distinguish in the Merino than in some other mammals because Henle's layer is not yet refractile, but the elongated nuclei of this layer may be seen in the cells which form a cone in the deeper part of the hair follicle. The length of the dermal papilla is at least equal to its diameter, and is frequently greater. The deepest part of the sudoriferous gland sometimes has a small lumen. The arrector pili muscle is always present, usually as two strands of smooth muscle fibres extending from the upper part of the dermis to the ental side of the wool

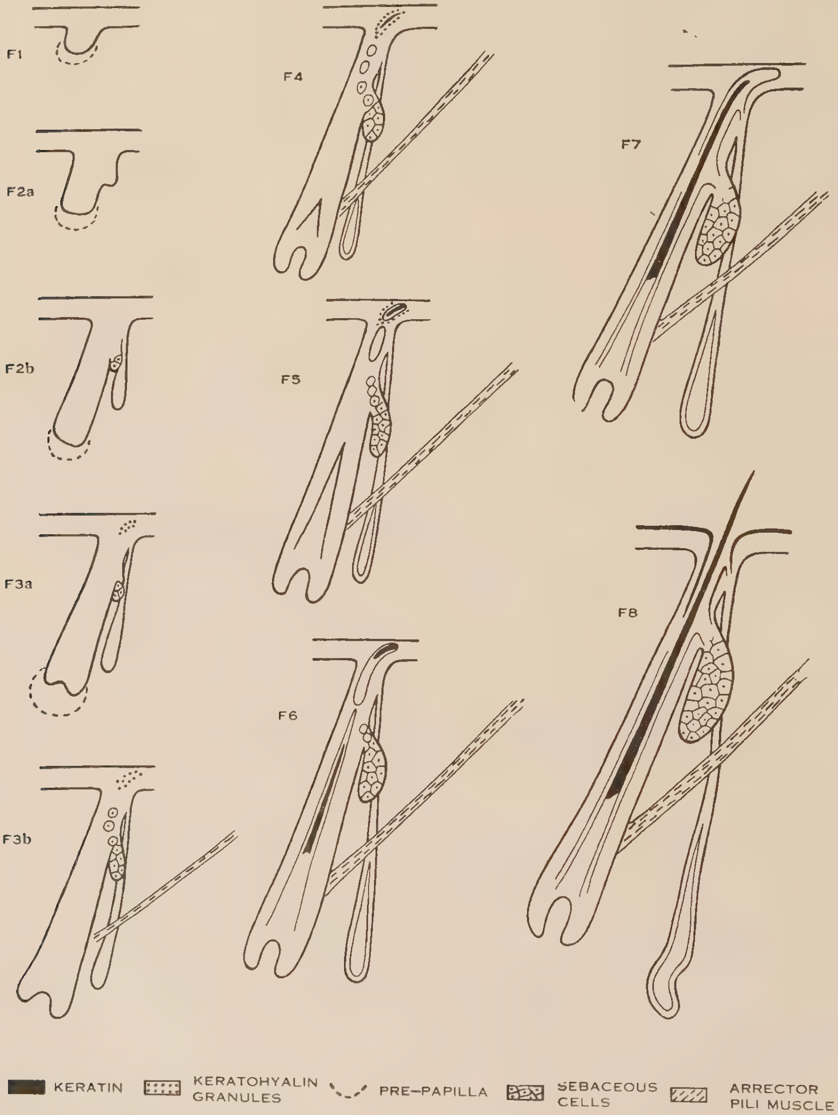


Fig. 2.—Diagram of stages *F*1–*F*8 in the development of primary wool follicles in the Merino foetus.

follicle at a deep level. In this region the outer root sheath sometimes has a marked ental swelling, as noted by Duerden and Ritchie (1924) and Auber (1950), but in many Merino foetuses a swelling is not apparent at this or later stages. The hair canal has developed further.

Stage F5.—The hair cone is now refractile and reaches the level of the base of the bilobed sebaceous gland, which is well developed. Inside the cone Huxley's layer and the developing hair cuticle and cortex can be recognized. The sudoriferous gland sometimes has a small lumen. There is frequently a hair canal space in both the epidermis and the follicle neck.

Stage F6.—The tip of the hair cone is solid and refractile, and lies at or above the level at which the sebaceous gland opens. The keratinized tip of the hair lies inside the cone. At this stage all the follicle layers can be recognized. The sudoriferous gland has usually begun to widen below the level where it passes between the two strips of the arrector pili muscle. Hair canal formation is completed.

Stage F7.—The tip of the wool fibre has emerged through the inner root sheath layers and now lies at the epidermal level in the hair canal on the ental side of the follicle. The uppermost part of the canal is usually bent, forming an obtuse angle with the axis of the follicle and lying almost parallel to the skin surface. The inner root sheath layers split and fragment as they reach the hair canal.

Stage F8.—The hair has pierced the uppermost layer of the epidermis (the periderm) and emerged.

(*b*) *Secondary Follicles*

There appears to be no complete account in the literature of the development of *S* follicles in sheep. Spöttel and Tänzer (1923) reported that in addition to the lack of sudoriferous glands and arrector pili muscles, there were several minor ways in which *S* follicles differed from *P* follicles in their development in Merino and English Blackface foetuses. However, they failed to report the arrangement of *S* follicles in bundles from the beginning of their development, which has been noted in the present study. In the Merino foetus most of the later *S* follicles, here to be referred to as derived secondary follicles (*SD*), were found to originate by simple branching from a few original secondary follicles (*SO*). A later paper by Tänzer (1926) on the Karakul contains what seems to be the only reference to a similar branching origin of *S* follicles in any breed.

The following is a brief account of the stages of development of the original secondary follicles, which are illustrated in Figure 3 and Plates 1 and 2.

Stage F1.—At the beginning of this stage an aggregation of dermal cells appears beneath the epidermal plug, which continues to elongate and retains its rounded base (Plate 1, Fig. 1; Plate 2, Fig. 1).

Stage F2.—The *SO* follicle is usually more elongated than the *P* follicles when its base begins to flatten (Plate 1, Fig. 2). A sudoriferous gland rudiment is not formed at this or any later stage. The sebaceous gland rudiment does not usually appear until later.

Stage F3.—As with *P* follicles, this stage can be subdivided into *F3a*, when the length of the papilla is less than its diameter (Plate 2, Fig. 2), and *F3b*, when its length is equal to or (rarely) greater than its diameter. The rudiment of the sebaceous gland, with differentiated sebaceous cells, usually appears at *F3a* (Plate 1, Fig. 3). No arrector pili muscles are formed at this or any later stage. At *F3b* a new (*SD*) follicle

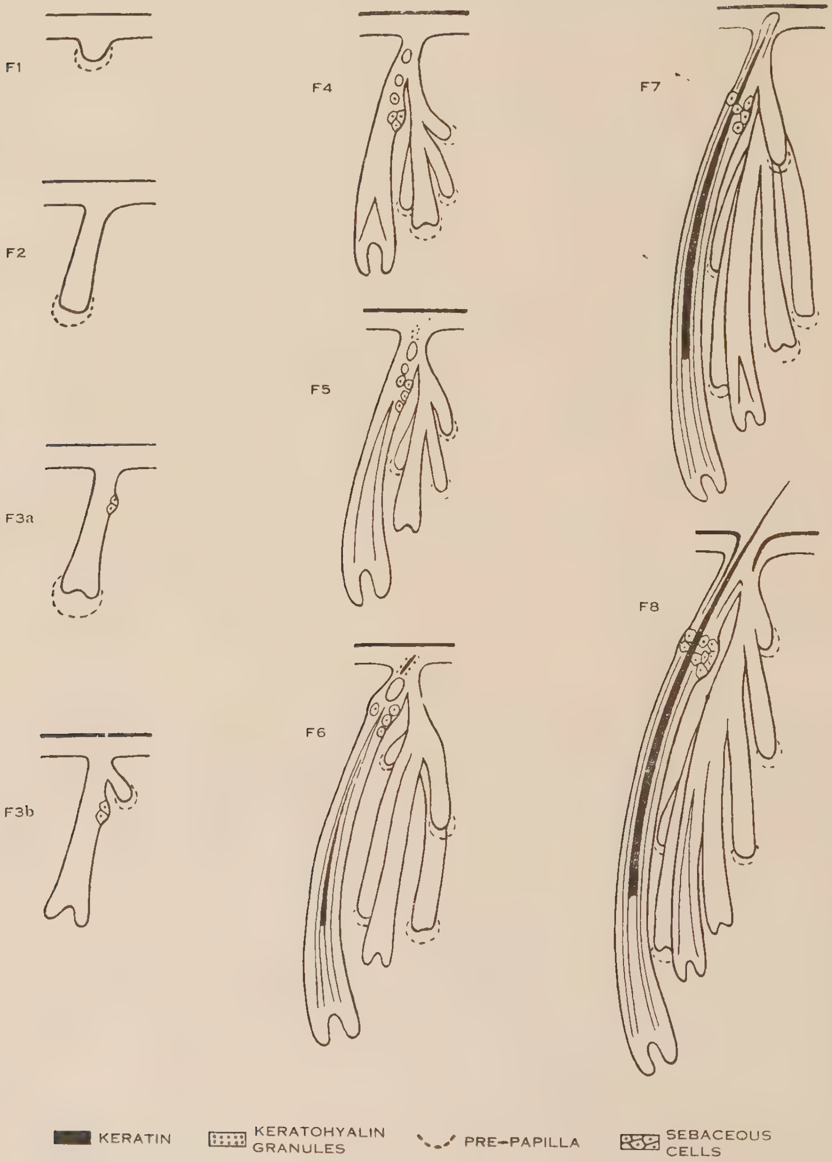


Fig. 3.—Diagram of stages *F*1–*F*8 in the development of original secondary follicles in the Merino foetus. Some stages in development of derived secondary follicles are also shown.

sometimes arises from the *SO* follicle, frequently as a lateral branch above the level of the sebaceous gland. Each *SD* follicle has at first a rounded base and would be classified as *F*1.

Stage F4.—The hair cone (Plate 2, Fig. 4) is similar to that of *P* follicles. Many differentiated sebaceous cells are present, but the gland rudiment does not protrude very far from the follicle wall, and is seldom bilobed. Sebaceous cells may migrate to the neck of the follicle prior to hair canal formation. An ental swelling of the outer root sheath, if present, is less conspicuous at all stages than in the *P* follicles. Frequently by this stage many *SD* follicles have formed as branches from the *SO* follicle or from the first *SD* follicle.

Stage F5.—The hair cone is refractile and reaches the level of the base of the sebaceous gland. Huxley's layer and the developing hair cuticle and cortex can be recognized inside the hair cone. The formation of the epidermal hair canal begins, frequently in the upper neck region of the follicle, and sebaceous cells are degenerating in the lower neck of the follicle. Further formation of *SD* follicles may occur.

Stage F6.—This stage is similar to that in *P* follicles except that hair canal formation is less advanced. There is usually a canal space in the lower neck of the follicle.

Stage F7.—The tip of the keratinized wool fibre has emerged through the inner root sheath and lies in a completed hair canal which is common to the bundle of follicles. The characteristic bending of the hair canal of *P* follicles was not observed in *SO* follicles. The inner root sheath layers are undergoing fragmentation as they reach the hair canal.

Stage F8.—The hair has emerged from the skin surface (Plate 1, Fig. 8).

The derived secondary follicles go through the same stages of development as the original ones, except for the following differences:

(i) Stage *F*1 is a budding from an *SO* follicle (Plate 1, Figs. 2 and 3; Plate 2, Fig. 3) or an *SD* follicle, and not from the epidermis.

(ii) The sebaceous gland may not form as early as stage *F*3. If it is not present before stage *F*5, the criterion for the latter stage would be the appearance of the hair cone at the deepest level of the sebaceous gland belonging to the *SO* follicle.

(iii) The hair canal into which the *SD* fibre enters at stage *F*7 is the common canal for the follicle bundle which was formed in advance of the tip of the fibre from the *SO* follicle.

The different types of branching of *S* follicles are best illustrated by diagrams. Some of the *SO* follicles, particularly some of the earliest to develop, never have branches (Fig. 1 (*b*)). These are called the unbranching original secondary follicles (*SOU*) to distinguish them from other branching original secondary follicles (*SOB*). Some branching appears at first as bifurcation, although lateral branching is more common (Fig. 3, *F*3*b*). Branching is usually at or above the level of the sebaceous gland (Figs. 4 (*a*) and 4 (*b*); Plate 1, Figs. 3 and 6), but is occasionally below it (Fig. 4 (*b*)). The largest bundles are usually those in which the *SOB* follicle is at the secondary margin of the follicle group, and the *SD* follicles arise in sequence as lateral branches from the *SOB* follicle or from one another, mainly in the direction of the

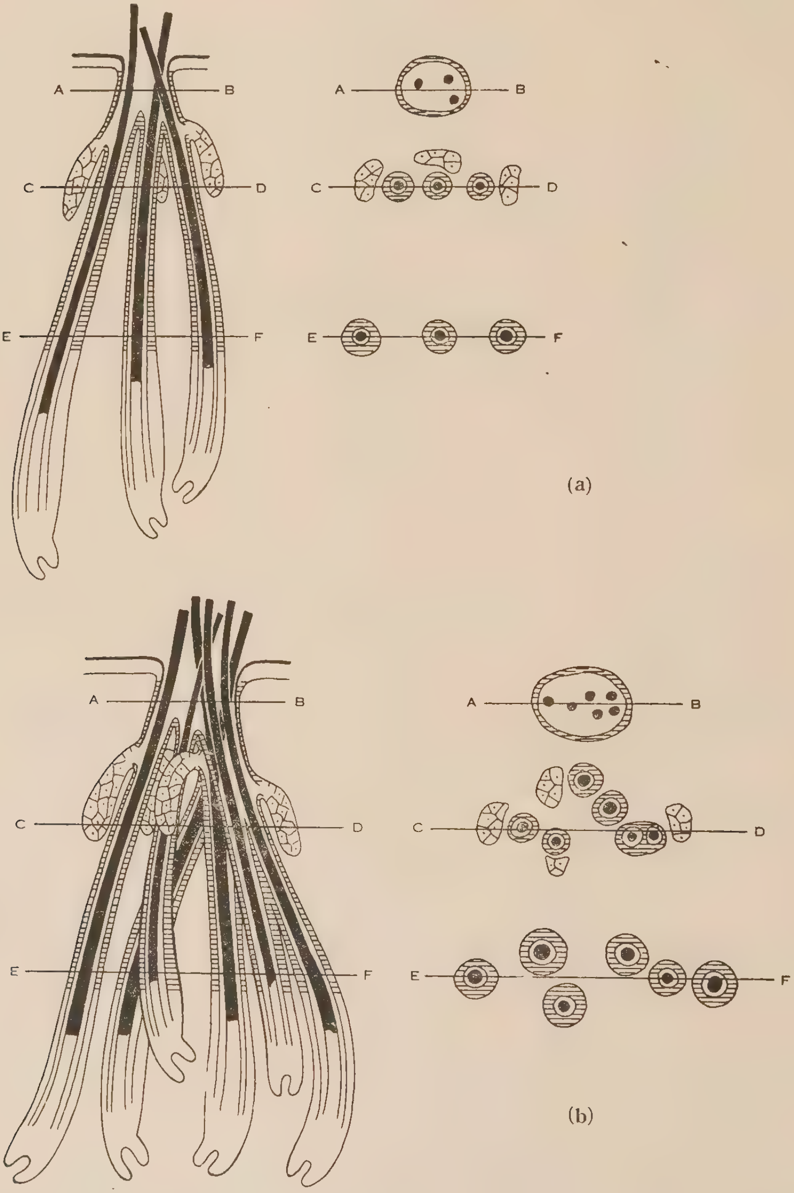


Fig. 4.—(*a*) Diagrammatic longitudinal and transverse sections through a bundle of three fully developed secondary follicles in the Merino. (*b*) Diagrammatic longitudinal and transverse sections through a bundle of six fully developed secondary follicles in the Merino.

primary margin of the group. Other bundles, in which the *SOB* follicle is nearer the centre of the group, tend to have fewer follicles.

By the end of pre-natal life at approximately 150 days, the branching of *S* follicles is extensive. As many as nine follicles have been found in one bundle with a common opening, and sometimes more than one of these is at stage *F*8, that is, a few *SD* follicles may have produced fibres. A full description of post-natal development will not be given here, but bundles of varying numbers of *S* follicles with a common funnel have been found at all ages examined, and these would seem to originate from the *SOB* follicles. In older sheep the common funnels were sometimes shorter than in foetuses and lambs, and sometimes had fewer fibres emerging from them. Shallow depressions on the skin surface with a large number of follicles opening into them were frequently observed.

IV. The Development of Follicle Groups

The description of 18 stages in the development of the follicle group in the Merino given by Carter (1943) was based mainly on the appearance of the groups in transverse sections at the level of the sebaceous glands. In the present work, Carter's sequence of group stages was confirmed in general, but since other levels and planes of section were studied, a more precise description of group stages is now possible.

The following, then, is a re-definition of the first 18 group stages proposed by Carter. The prefix *G* is added to the stage numbers to distinguish them from the *F* stages of individual follicles described above. The criteria for distinguishing each stage, printed in italics, are those which Carter and Hardy (1947) actually employed, although they did not state the fact. This description should be read in conjunction with Table 1, which indicates the *most advanced* stage of development which was *usually* found in each type of follicle at each group stage.

Stage G1: *Central primary* X *follicles* (PCX) *present.*—These are more or less equally spaced on the skin surface.

Stage G2: *Central primary* X *and early central primary* Y *follicles* (PCY) *present.*—*PCX* follicles have increased in length but are still at *F*1: they already have a characteristic slope, and later follicles within one region usually grow with approximately the same angle of slope. The *PCY* follicles, which are found in the spaces between the *PCX* follicles, are smaller than the latter.

Stage G3: *Sudoriferous gland buds formed with central primary* X *follicles.*—*PCX* follicles are at *F*2*a*.

Stage G4: *Trio groups formed by the addition of a lateral primary* x *follicle* (PLx) *on each side of some of the central primary* X *follicles.*—These trios of primary follicles constitute the primary margins of the groups that will form later.

Stage G5: *Further trio groups formed by the addition of some lateral primary* y *follicles* (PLy) *on either side of the central primary* Y *follicles, as well as trios with the central primary* X *follicles.*

Stage G6: *Sudoriferous gland buds formed in association with all types of primary follicles.*—*PCX*, *PCY*, *PLx*, and *PLy* follicles are at *F*2*a*. The linear arrangement of follicles is now clear. In addition to trios, some solitary (*PCX* or *PCY*) or couplet (*PCX*, *PLx*; or *PCY*, *PLy*) groups are found.

Stage G7: *Sebaceous gland buds formed on the ental side of central primary follicles* (PCX *and* PCY).—*PCX* follicles are at *F*3*a* or *F*2*b*, and *PCY* follicles at *F*2*b*.

Stage G8: *Secondary follicle formation begun.*—The first plugs (*F*1) of *SO* follicles appear between the *PC* and *PL* follicles on their ectal side.

Stage G9: *Sebaceous gland buds formed on the ental side of some lateral primary follicles* (PLx *and* PLy).—*PLx* follicles are at *F3a* or *F2b*, and *PLy* follicles at *F2b* or *F2a*. There is an increased number of *SO* follicles. The first-formed *S* follicles tend to define the secondary margin of the group, and later *S* follicles form between them and the *P* follicles.

TABLE 1

SUMMARY OF STAGES IN THE DEVELOPMENT OF FOLLICLE GROUPS AND THEIR RELATION TO THE GROWTH OF INDIVIDUAL FOLLICLES

Mean Age of Foetus* (days)	Follicle Group Stage	Most Advanced Stages Found in Each Follicle Type†				
		PCX	*PCY*	*PLx*	*PLy*	*SO*
64	*G*1	**F1**	—	—	—	—
69	*G*2	*F*1	**F1**	—	—	—
71	*G*3	**F2a**	*F*1	—	—	—
74	*G*4	*F2a*	*F2a*	**F1**	—	—
76	*G*5	*F2a*	*F2a*	*F2a*, *F*1	**F1**	—
79	*G*6	*F2a*	*F2a*	**F2a**	**F2a**	—
82	*G*7	*F3a*, **F2b**	*F2b*	*F2a*	*F2a*	—
86	*G*8	*F3b*, *F3a*	*F2b*	*F2a*	*F2a*	**F1**
90	*G*9	*F3b*, *F3a*	*F3b*, *F3a*	*F3a*, **F2b**	*F2b*, *F2a*	*F*1
95	*G*10	**F4**	*F*4	*F3b*, *F3a*	*F3a*	*F*2
97	*G*11‡	*F*5	*F*5	*F3b*	*F3a*	*F*2
98	*G*12	*F*6	*F*6, *F*5	*F*5, **F4**	*F*4, *F3b*	*F*2
99	*G*13	*F*6	*F*6	*F*5	*F*5, *F*4	*F3b*, **F3a**
101	*G*14	**F7**	*F*7	*F*7, *F*6	*F*7, *F*6	*F3b*, *F3a*
102	*G*15	**F8**	*F*8	*F*7	*F*7, *F*6	*F3b*
107	*G*16	*F*8	*F*8	**F8**	*F*8, *F*7	*F*4, *F3b*
115	*G*17	*F*8	*F*8	*F*8	*F*8	*F*7, **F6**
133	*G*18	*F*8	*F*8	*F*8	*F*8	**F8**

* From the study of the relation between age of foetus and stage of development by Carter and Hardy (1947). These figures may be interpreted also as representing the approximate age for each stage on the mid-side region.

† Heavy type indicates those follicle stages which define the group stages. Where two follicle stages are shown for a follicle type at a single group stage, some skin samples contain the more advanced follicle stage indicated, while others contain only the slightly less advanced stage.

‡ *G*11 is defined not by a follicle stage but by a stage in hair canal formation.

Stage G10: *Hair cones in some central primary follicles* (PCX *and* PCY).—Some *PCX* and *PCY* follicles are at *F*4, with rudimentary arrector pili muscles. *PLx* follicles are at *F3b* (some with rudimentary arrector pili muscles) or *F3a*.

Stage G11: *Hair canal spaces formed in the epidermis above central primary follicles* (PCX *and* PCY).—*PCX* and *PCY* follicles are at *F*5 (with arrector pili muscles) and the *PLx* follicles are at *F3b*, some with arrector pili muscles.

Stage G12: *Hair cones in lateral primary follicles* (PLx *and* PLy).—Some of the *PC* follicles are at *F*6. In material stained with picric acid, the appearance of keratinized fibres in these follicles is the best criterion for this stage. The *PLx* follicles are at *F*5 or *F*4 with arrector pili muscles, and the *PLy* follicles at *F*4 (with arrector pili muscles) or *F3b*.

Stage G13: *Sebaceous gland buds formed on some of the secondary follicles.*—The first-formed *SO* follicles, which are at *F3b* or *F3a*, are the first to have sebaceous gland buds. *SD* follicles may appear at this stage. There is sometimes a hair canal space in the epidermis above the *PL* follicles.

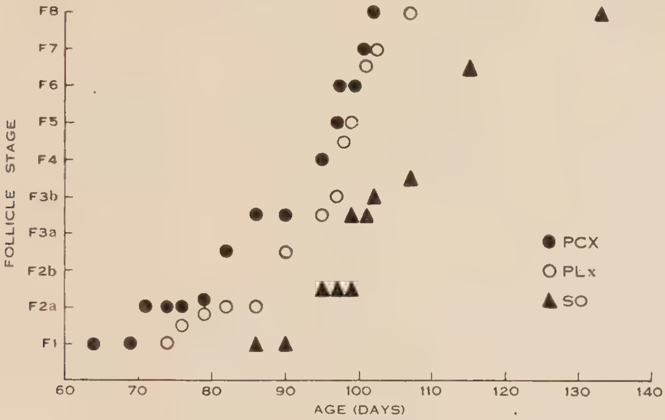


Fig. 5.—Graph showing the relation between the mean age for each group stage and the most advanced follicle stage found among central primary *X* (*PCX*), lateral primary *x* (*PLx*), and original secondary (*SO*) follicles respectively. The mean age for each group stage was obtained from the data of Carter and Hardy (1947). The group stage at each of 12 sampling points on 12 foetuses was plotted against the age of each foetus and from a smoothed curve the mean age for each group stage was read.

Stage G14: *Central primary follicles have fibres in hair canals at the level of the epidermis.*—*PCX* and *PCY* follicles are at *F*7.

Stage G15: *Central primary fibres have pierced the periderm and emerged from the skin surface.*—*PC* follicles are at *F*8. *SO* follicles show much branching.

Stage G16: *Lateral primary fibres have pierced the periderm and emerged.*—All types of *P* follicles have reached *F*8 except the *PLy* follicles which are sometimes not beyond *F*7.

Stage G17: *Early secondary fibres formed.*—All types of *P* follicles have reached *F*8. *SO* follicles have reached *F*6, and sometimes *F*7. Bundles of up to nine *S* follicles are found. The ratio of the number of *S* to the number of *P* fibres (*Sf*/*Pf*) at the level of the sebaceous glands is less than 2·0.

Stage G18: *Early secondary fibres emerged.*—The tips of the first *SO* fibres have passed through a hair canal and emerged. The *Sf*/*Pf* ratio is between 2·0 and 2·9.

By making use of the mean age for each group stage, estimated from 12 sampling points on the fleece-bearing area in the G series of foetuses by Carter and Hardy (1947, p. 12 and Fig. 2), the approximate times and rates of development of the different follicle types can now be compared (Table 1).

The relation between the *mean* age for each group stage and the *most advanced* follicle stage found among *PCX*, *PLx*, and *SO* follicles respectively is shown in Figure 5. In this graph the arbitrary follicle stages and sub-stages are placed at equal intervals along the ordinate so that the actual shape of a curve fitting the points plotted is not of biological significance in itself. The shape of the "stage-age curve" is similar for *PCX* and *PLx* follicles. The *PCY* follicles begin slightly later than *PCX* follicles but after about the 90th day of gestation their development almost coincides. Similarly the *PLy* follicles begin a little later than *PLx* follicles but soon catch up. The stage-age curve for the *earliest SO* follicles is similar in shape to those for the *P* follicles in the initial stages of development; the apparent slowing between stages *F*6 and *F*8 is probably spurious, due to the inaccurate estimates of mean age resulting from a small number of older foetuses in the G series.

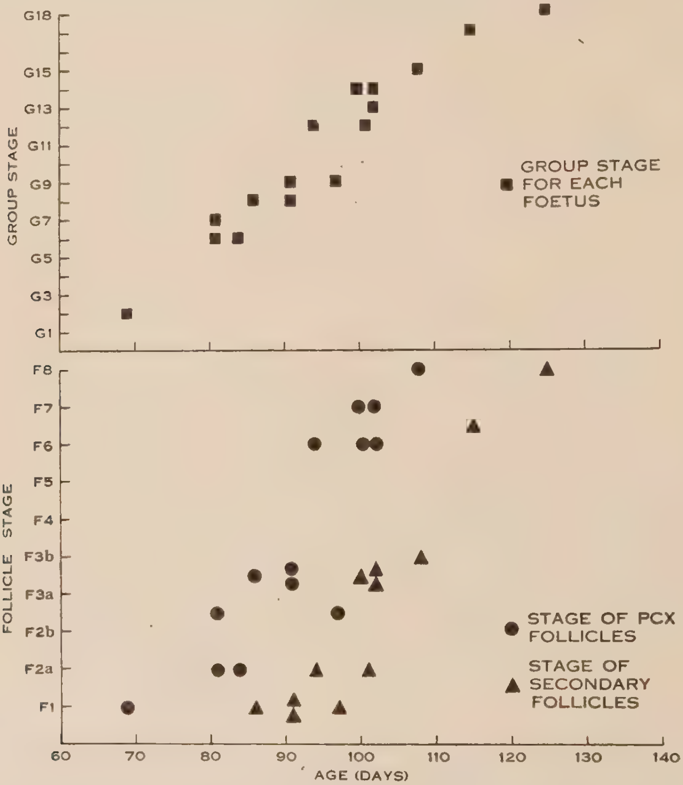


Fig. 6.—Graph showing the relation in the dorsal posterior thoracic region (see text) of (i) stages in development of follicle groups, and (ii) stages in development of central primary *X* (*PCX*) and original secondary (*SO*) follicles, to the actual age of each foetus in the G and KR series.

Figure 6 shows the stages of development of follicle groups and of *PCX* and *SO* follicles on the dorsal posterior thoracic region (D1–D2, Carter and Hardy 1947) in relation to the *actual* (recorded or estimated) age of each foetus in the G and KR series. No marked difference in shape between the stage-age curves of *PCX* and *SO* follicles could be detected. However, it must be emphasized that the present observations are restricted to the earliest *SO* follicles, and that later *S* follicles may not have the same rate of development.

V. DISCUSSION

From the data of Carter and Hardy (1947) and the present study (Figs. 5 and 6) it was estimated that the development from stage *F*1 to stage *F*8 in *P* follicles occupied about 38 days, and in the first *SO* follicles occupied about 40 days. Less is known of the rate of development of *SD* follicles. Most of the secondaries are probably initiated by about 135 days of gestation (Carter and Hardy 1947) and the remainder before birth (Schinckel 1955*b*; Short 1955*a*). As the majority have reached at least *F*6 by 28 days after birth (Fraser 1954; Schinckel 1955*a*; Short 1955*a*), it is probable that most *SD* follicles take between 30 and 45 days to develop from *F*1 to *F*8. However, the small number which do not produce fibres until 4 or 6 months after birth (Schinckel 1955*a*; Short 1955*a*) must take considerably longer to develop.

The origin of later *S* follicles by branching from earlier ones was first reported in the Karakul by Tänzer (1926). Frölich, Spöttel, and Tänzer (1929), without citing a reference, indicated that additional observations had been made by Tänzer on follicle branching in other breeds, including the Merino. None of the later writers referred to Tänzer's work, which contradicted the previous conclusions of Spöttel and Tänzer (1923). Duerden (1939) considered that *S* follicles were formed from a wedge of undifferentiated epidermal tissue which later became divided laterally into separate follicles. In the legend to his Plate 1, Figure 5, illustrating skin from a 16-week Romney Marsh foetus, he expressly stated "The follicles of a group appear as if arising by branching or budding; this is not so, but each group contains several closely adjacent follicles." Carter (1943) followed Duerden in his description of *S* follicle development. Although it is possible that some of the later formed *S* follicles may arise in the manner described by Duerden, there is strong evidence from the present study that the majority arise by simple budding from other discrete *S* follicles which are already undergoing differentiation. This conclusion was reached independently of that of Tänzer, whose findings were not available to the present authors until the completion of their observations.

It is remarkable that none of the more recent studies of the development of follicle populations have included any reference to branching follicles, although they are apparent in new-born Merino lambs; perhaps this is due to preoccupation with transverse sections through the follicles at the level of the sebaceous glands. In the following paragraphs the relevance of this finding to the current studies will be outlined.

It is generally agreed that wool fibre density (that is the number of fibres per unit area), which is strongly inherited, is one of the most important components of fleece structure. In the Merino the total number of *P* fibres is fairly constant at different ages and between individuals (Carter and Hardy 1947; Schinckel 1955*a*), but the total number of *S* fibres varies greatly between individuals and strains. Thus the ratio *Sf*/*Pf*, which may range from 10 to 46 (Carter and Clarke, personal communication) is the most useful measure of differences in fibre numbers. The ratio of the total numbers of secondary follicles to primary follicles, or *S*/*P*, which is a measure of the potential number of wool fibres that can appear under favourable conditions, seems to be maximal at birth (Carter and Hardy 1947; Schinckel 1955*b*; Short 1955*a*). Variations in this most important determinant of wool fibre density in the adult Merino are now known to be largely due to two factors; these are the number of *SO* follicles and the number of *SD* follicles formed from them. There is no reason to assume *a priori* that the two processes are controlled in exactly the same way, and they need to be examined separately. No correlation has been found between *S*/*P* and birth weight (Schinckel 1955*b*; Short 1955*a*) and no significant difference between *S*/*P* values in singles and twins (Schinckel 1955*b*). Adverse maternal nutrition also failed to influence this ratio (Short 1955*b*). Thus overcrowding of follicles in small lambs does not seem to limit the branching activity which occurs during the latter part of the gestation period when differences in body size become marked, in British breeds at least (Barcroft 1946).

Reported values for *Sf*/*Pf* in Merinos at birth range from 0·9 to 7·3 (Carter and Hardy 1947; Fraser 1954; Schinckel 1955*b*; Short 1955*a*), but are usually between 2 and 4. These are of the same order as the values for *SO*/*Pf* at birth, and it may be deduced that the majority of *S* fibres present are from *SO* follicles. Within a flock, *Sf*/*Pf* at birth, unlike *S*/*P*, was highly correlated with birth weight (Schinckel 1953, 1955*b*; Short 1955*a*) and was lowered by adverse pre-natal nutrition (Short 1955*b*) so that these factors must affect the rate at which *SO* follicles, and perhaps the first *SD* follicles, mature. The development of the rest of the *SD*, which comprise most of the immature follicles present at birth, is apparently affected by growth and nutrition in early post-natal life, since *Sf*/*Pf* in adults was positively correlated with birth weight (Schinckel 1955*b*; Short 1955*a*) and growth from birth to 1 month (Schinckel 1955*b*) and was lowered by poor nutrition in the period immediately after birth (Short 1955*b*). A qualitative and quantitative study of *SO* and *SD* follicles in association with such investigations should be fruitful.

The distinction between *SO* and *SD* follicles may also throw some light on differences between breeds and strains. Branching is known to occur prenatally in the Karakul (Tänzer 1926) as well as the Merino, and Duerden's (1939) illustration suggests to the authors that it may occur in the Romney. *S* follicle bundles with common openings were found in the adults of all the many breeds examined by Spöttel and Tänzer (1923), but there was a progressive increase in the number of bundles and the number of follicles per bundle from the primitive Mufflon to the fine-woolled Merino. Some *S* follicle bundles in lambs or adults were also noted in the Romney by Burns and Clarkson (1949), in the South African Merino by Duerden and Ritchie (1924), and in the Corriedale as well as the Merino by Hardy and Lyne (unpublished observations). Although Spöttel and Tänzer attributed the increase in bundle formation from the Mufflon to the Merino to the increasing convergence of follicles at the skin surface, with consequent adhesion and fusion, it may well be due rather to an increased amount of branching in pre-natal life. Since the high *Sf/Pf* ratio is the most outstanding feature which distinguishes the Merino from other breeds (Carter 1942, 1955), it is important to know how far this is due to an increase in the number of *SO* follicles and how far to an increase in the extent of branching. The same argument applies to the differences between strains within the Merino breed. Other important distinctions between strains and breeds are concerned with the types of fibres produced by different types of follicles (Carter 1955). Although the *S* fibres at the margin of a group, which are usually the first to be formed, tend to be thicker and more frequently medullated than those towards the centre (Carter 1943), and their follicles may produce different fibre types in lambs (Fraser 1952*a*), there has hitherto been no clear anatomical basis for distinguishing two types of *S* follicles. Fraser (1953) postulated that two waves of *S* follicle development, occurring before and after birth respectively, may have different levels of efficiency in fibre production. Making use of a theory of competition for "fibre substrate" (Fraser 1951; Fraser and Short 1952) and the concept of different efficiency levels for *P*, first-wave *S*, and second-wave *S* follicles, Fraser (1953) sought to explain the differences in fleece structure between breeds. His two efficiency levels for *S* follicles may apply to the *SO* and *SD* follicles respectively; this is another promising subject for investigation.

VI. ACKNOWLEDGMENTS

The authors are indebted to the following for the loan or supply of material: Mr. H. B. Carter, formerly of the Division of Animal Health and Production, C.S.I.R.O., and now on the staff of the Animal Breeding Research Organization, Edinburgh; Dr. B. F. Short, Sheep Biology Laboratory, Division of Animal Health and Production, C.S.I.R.O., Prospect, N.S.W. The assistance of Miss M. J. Heideman in the preparation of sections and the tracing of complete follicle groups and of Mr. I. T. Roper in the preparation of photographs is gratefully acknowledged.

VII. REFERENCES

AUBER, L. (1950).—The anatomy of follicles producing wool-fibres, with special reference to keratinization. *Trans. Roy. Soc. Edin.* **62**: 191–254.

BARCROFT, J. (1946).—"Researches on Pre-Natal Life." Vol. 1. (Blackwell Scientific Publications Ltd.: Oxford.)

BURNS, MARCA (1953).—Observations on the follicle population of Blackface sheep. *J. Agric. Sci.* **43**: 422–31.

BURNS, MARCA (1954*a*).—Observations on the development of the fleece and follicle population in Suffolk sheep. *J. Agric. Sci.* **44**: 86–99.

BURNS, MARCA (1954*b*).—The development of the fleece and follicle population in Herdwick sheep. *J. Agric. Sci.* **44**: 443–64.

BURNS, MARCA, and CLARKSON, HILDA (1949).—Some observations on the dimensions of follicles and of other structures in the skin of sheep. *J. Agric. Sci.* **39**: 315–34.

CARTER, H. B. (1942).—"Density" and some related characters of the fleece in the Australian Merino. *J. Coun. Sci. Industr. Res. Aust.* **15**: 217–20.

CARTER, H. B. (1943).—Studies in the biology of the skin and fleece of sheep. 1. The development and general histology of the follicle group in the skin of the Merino. Coun. Sci. Industr. Res. Aust. Bull. No. 164.

CARTER, H. B. (1955).—The hair follicle group in sheep. *Anim. Breed. Abstr.* **23**: 101–16.

Carter, H. B., and Hardy, Margaret H. (1947).—Studies in the biology of the skin and fleece of sheep. 4. The hair follicle group and its topographical variations in the skin of the Merino foetus. Coun. Sci. Industr. Res. Aust. Bull. No. 215.

Cloete, J. H. L. (1939).—Prenatal growth in the Merino sheep. *Onderstepoort J. Vet. Sci.* **13**: 417–558.

Duerden, J. E. (1939).—The arrangement of fibre follicles in some mammals, with special reference to the Ovidae. *Trans. Roy. Soc. Edin.* **59**: 763–71.

Duerden, J. E., and Ritchie, M. I. F. (1924).—Development of the Merino wool fibre. *S. Afr. J. Sci.* **21**: 480–97.

Fraser, A. S. (1951).—Competition between skin follicles in sheep. *Nature* **167**: 202–3.

Fraser, A. S. (1952*a*).—Growth of wool fibres in sheep. *Aust. J. Agric. Res.* **3**: 419–34.

Fraser, A. S. (1952*b*).—Growth of the *N*-type fleece. *Aust. J. Agric. Res.* **3**: 435–44.

Fraser, A. S. (1953).—Factors in the genetic determination of fleece structure in sheep. *J. Genet.* **51**: 222–36.

Fraser, A. S. (1954).—Development of the skin follicle population in Merino sheep. *Aust. J. Agric. Res.* **5**: 737–44.

Fraser, A. S., and Short, B. F. (1952).—Competition between skin follicles in sheep. *Aust .J. Agric. Res.* **3**: 445–52.

Frölich, G., Spöttel, W., and Tänzer, E. (1929).—"Wollkunde." (Julius Springer: Berlin.)

Hardy, Margaret H. (1949).—The development of mouse hair *in vitro* with some observations on pigmentation. *J. Anat., Lond.* **83**: 364–84.

Hardy, Margaret H., and Lyne, A. G. (1956*a*).—The histological development of the skin and wool in the Merino foetus. *Proc. Int. Wool Text. Res. Conf. Aust.* **F**: 26–31.

Hardy, Margaret H., and Lyne, A. G. (1956*b*).—The development of wool follicles from foetal sheep skin in tissue culture. *Proc. Int. Wool Text. Res. Conf. Aust.* **F** : 32–5.

Hardy, Margaret H., and Lyne, A. G. (1956*c*).—Proposed terminology for wool follicles in sheep. *Nature* **177** : 705-6.

Margolena, L. A. (1954).—Sequence and growth of primary and secondary fiber follicles in Karakul sheep. *J. Anim. Sci.* **13**: 765–80.

Schinckel, P. G. (1953).—Follicle development in the Australian Merino. *Nature* **171**: 310.

Schinckel, P. G. (1955*a*).—The post-natal development of the skin follicle population in a strain of Merino sheep. *Aust. J. Agric. Res.* **6**: 68–76.

Schinckel, P. G. (1955*b*).—The relationship of skin follicle development to growth rate in sheep. *Aust. J. Agric. Res.* **6**: 308–23.

Short, B. F. (1955*a*).—Development of the secondary follicle population in sheep. *Aust. J. Agric. Res.* **6**: 62–7.

Short, B. F. (1955*b*).—Developmental modification of fleece structure by adverse maternal nutrition. *Aust. J. Agric. Res.* **6**: 863–72.

Spöttel, W., and Tänzer, E. (1923).—Rassenanalytische Untersuchungen an Schafen unter besonderer Berücksichtigung von Haut und Haar. *Arch. Naturgesch.* **89**: 1–242.

Tänzer, E. (1926).—"Haut und Haar beim Karakul in rassenanalytischen Vergleich." (Otto Thiele: Halle (Saale).)

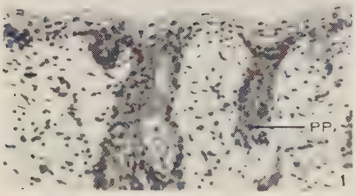
Wildman, A. B. (1932).—Coat and fibre development in some British sheep. *Proc. Zool. Soc. Lond.* **2**: 257–85.

Wildman, A. B., and Carter, H. B. (1939).—Fibre-follicle terminology in the Mammalia. *Nature* **144**: 783–4.

Explanation of Plates 1 and 2

All figures are of sections through skin from the trunk of Merino foetuses. The staining is with haemalum, eosin, and picric acid, except Plate 1, Fig. 4, which is without picric acid. *DP*, dermal papilla; *G*, stratum germinativum; *H*, hair; *HN*, hair cone; *P*, periderm; *PP*, pre-papilla; *S*, stratum spinosum; *SD*, derived secondary follicle; *SG*, sebaceous gland; *SO*, original secondary follicle.

DEVELOPMENT OF WOOL IN MERINO SHEEP



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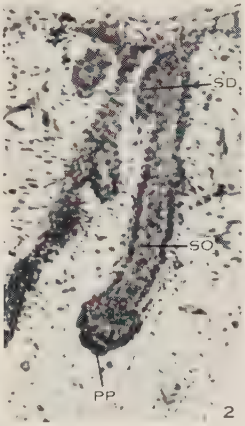

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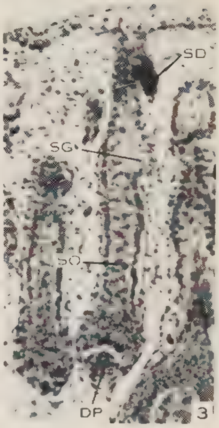

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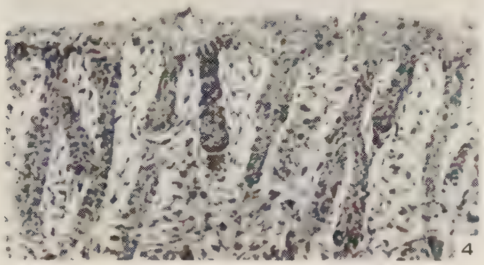

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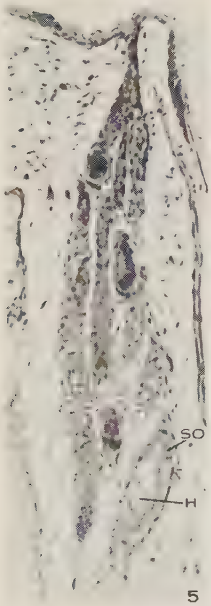

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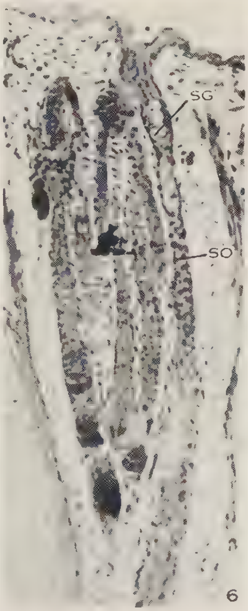

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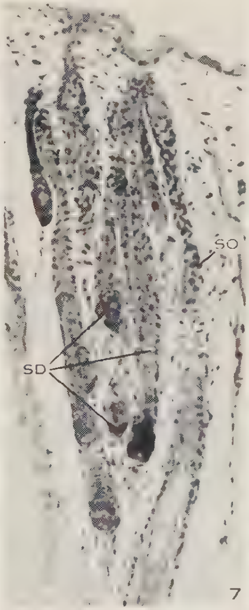

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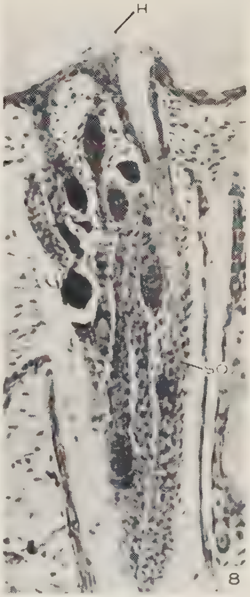

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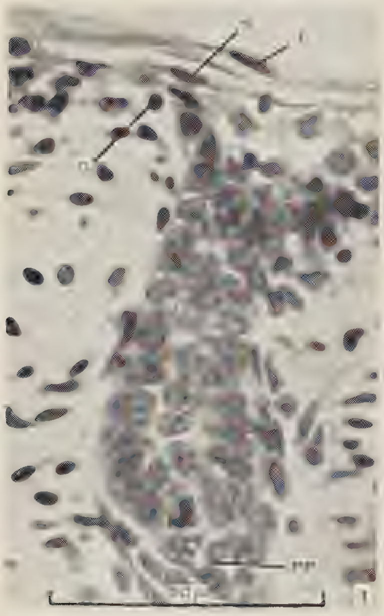

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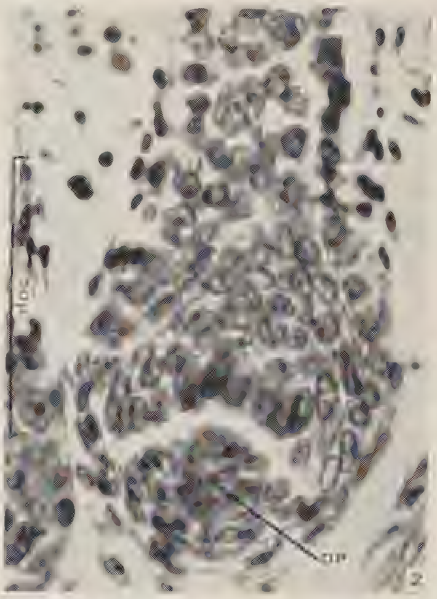

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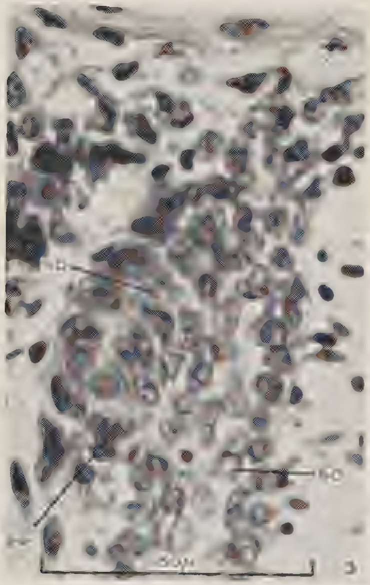

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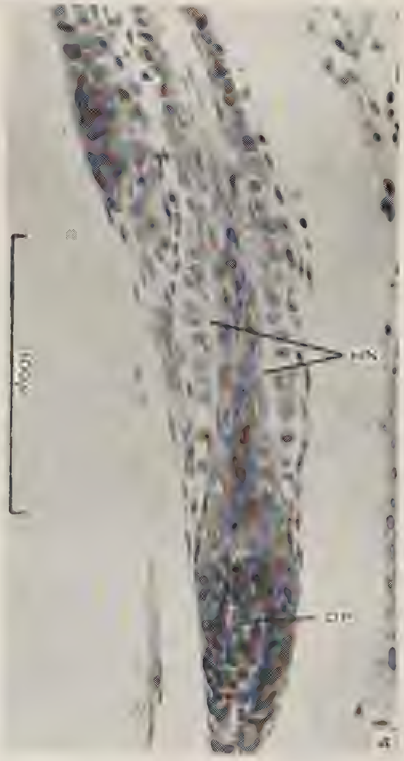

4

Plate 1

Development of branching secondary follicles

Fig. 1.—Vertical section of skin from 98-day foetus with two *S* follicles at stage *F*1.

Fig. 2.—Vertical section of skin from 98-day foetus with an *SO* follicle at stage *F*2 and an *SD* follicle at stage *F*1.

Fig. 3.—Vertical section of skin from 98-day foetus with an *SO* follicle at stage *F*3*a* and an *SD* follicle at stage *F*1.

Fig. 4.—Oblique section of skin from 102-day foetus with *S* follicles which show branching.

Fig. 5.—Longitudinal section of bundle of *S* follicles from 125-day foetus showing the narrow neck of the bundle. Oblique section of portion of *SO* follicle at stage *F*6.

Fig. 6.—Longitudinal section of two adjacent bundles of *S* follicles from 125-day foetus. *SO* follicle of bundle on right at stage *F*6.

Fig. 7.—Section adjacent to that shown in Figure 6.

Fig. 8.—Longitudinal section of bundle of *S* follicles from 125-day foetus with *SO* follicle at stage *F*8. Three *S* follicles belonging to another bundle are seen on the left.

Plate 2

Development of secondary follicles

Fig. 1.—Longitudinal section of an *SO* follicle at stage *F*1 from 98-day foetus.

Fig. 2.—Base of *SO* follicle at stage *F*3*a* shown in Plate 1, Figure 3.

Fig. 3.—*SD* follicle at stage *F*1 shown in Plate 1, Figure 2.

Fig. 4.—Longitudinal section of lower part of an *SO* follicle at stage *F*4 from 98-day foetus.

MAST CELLS AND HAIR GROWTH IN THE MOUSE

By T. Nay*

[*Manuscript received January* 24, 1956]

Summary

(i) In the skin of Naked, Crinkled, and occasionally, normal mice, the mast cell granules, or whole mast cells, located in subdermal connective tissue, become pigmented, the colour being the same as the colour of the hair of the animal in question.

(ii) In the skin of Crinkled mice, which have little hair growth, a great number of pigmented mast cells accumulate in the subdermal connective tissue.

(iii) The pigmented mast cell granules in Naked and Crinkled mice are engulfed by the growing hair follicle, and accumulated in the dermal papilla.

(iv) After the formation of pigment in the hair the dermal papilla appears depleted of pigmented granules.

(v) It is suggested that mast cells in the mouse are concerned in pigment formation.

I. Introduction

Since the discovery of mast cells by Ehrlich (1877) two important facts about their physiological function have been established. Holmgren and Wilander (1937), Jorpes, Holmgren, and Wilander (1937), and Hirt (1938), showed that the amount of heparin extracted from a tissue is proportional to the number of mast cells contained in it, indicating that the mast cells are the source of heparin. Riley (1953*a*, 1953*b*) and Riley and West (1953) found a correlation between the histamine content of normal and pathological tissues and the number of mast cells contained in them. Mast cell tumors proved to be extremely rich in histamine (Riley 1953*a*). But, as Riley (1953*a*) points out, "it seems possible, that the production of heparin and histamine are part of the mast cell function, and that there remains unproved a third function, which is probably a fundamental interaction between the mast cells and the connective tissue." The present paper is concerned with the relation of mast cells to the hair growth cycles.

II. Material and Methods

Over 100 mice were used in the course of the investigation. Of these, 25 were from the CR stock, which is maintained segregating for the *cr* (crinkled) gene (Grüneberg 1952), and the rest from the NM stock, which is maintained segregating for the *N* (Naked) gene (Grüneberg, loc. cit). All mice were bred and maintained at the Animal Genetics Section, C.S.I.R.O., University of Sydney.

The mice were skinned immediately after killing, the skins pinned on a cork frame, fixed in 70 per cent. alcohol, dehydrated, and cleared in xylol in order to remove fat; the skins were then passed through alcohol to water, stained for a few minutes in 0·25 per cent. methylene blue, dehydrated, cleared again, and mounted in "Siro". This treatment stains the hair follicles and muscles light blue, the connective tissue remaining practically unstained; the mast cells and their scattered granules are stained deep purple (Zahl and Nowak 1949). It is unfortunate that the growing follicles stain and obscure the view. The area of skin examined was a dorsal strip between neck and tail root, about 2 cm wide.

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* Animal Genetics Section, C.S.I.R.O., University of Sydney.

In course of the work, a new and simple method of preparing and staining mast cells was developed. Whole skins were fixed in 5 per cent. formol-saline to which about 1 per cent. iron oxide had been added. After 4–5 days in fixative, the skins were dehydrated, cleared in xylol, and mounted in "Siro". The mast cell granules stain selectively a bright straw-yellow colour, the rest of the tissue stains a uniform pale yellow.

Selected areas from the cleared skins were embedded in paraffin, cut serially at 20 μ, and mounted on slides in the usual way. Some paraffin sections were stained in 0·5 per cent. thionin dissolved in 20 per cent. alcohol; some were examined unstained for purposes explained in the next section.

The reason for using Naked mice is that in the skin of these mice it is sometimes possible to examine two complete hair growth cycles on one slide, with hair follicles in every desired stage of growth from anagen to telogen. The stages of the development of hair follicles in the mouse have been described in detail by Dry (1926).

III. Results

The main observations were as follows:

(i) In the areas of the skin where hair growth is in the resting phase, a high density of mast cells can be seen, many of them disintegrating and scattering their granules into the surrounding connective tissue.

(ii) The number of mast cells appears to be less in areas in active hair growth, an impression which may be misleading, because some of the mast cells in the dermis may be concealed by bulky hair follicles. Apart from this doubtful quantitative difference, there is clearly a difference in the shape of the mast cells in regions actively in the growth phase compared to regions in the resting phase. Mast cells in growing regions are compact and sharply outlined, and only occasionally can a disintegrating cell be found.

(iii) In areas of skin in which the follicles are in the early anagen stage, mast cells or their isolated granules can occasionally be found which do not stain purple but appear to be black, brown, or light brown, depending on the colour of the animal. In some preparations from black animals, whole nests of mast cells were observed which had turned black in colour. The phenomenon is not due to treatment, because the black mast cells can be seen under a dissecting microscope within a few minutes of killing the animal, and identified as bluish spots immediately after skinning. Most of the pigmented granules appear below, around, or clinging to the outer side of the dermal papilla, and in the loose connective tissue between the growing follicle and the loose subdermal connective tissue (see Plate 1, Fig. 1; Plate 2, Figs. 2, 3, and 4; Plate 3, Figs. 1, 2, and 3). Cells entirely filled with pigmented granules are always centered below the hair follicles. In iron oxide preparations, some cells can be seen in which the granules are of both kinds, some being straw-yellow and some pigmented.

This suggests that the cells with pigmented and non-pigmented granules are the same, the granules being converted into pigment in the pigmented cells.

In early anagen, the dermal papilla of the follicle is devoid of pigmented granules (Plate 2, Fig. 1). As the follicle grows, a few loose granules cling to the outside of the dermal papilla (Plate 2, Figs. 2 and 3), and at a later stage pass inside it and appear as a compact lump (Plate 2, Fig. 4). As the growth progresses, the lump of granules inside the dermal papilla becomes bigger and denser (Plate 3, Figs. 1 and 2) and the epidermal papilla starts to grow over it in an enveloping fashion (Plate 3, Figs. 3 and 4; Plate 4, Figs. 1 and 2). Finally, the whole lump is contained within the follicle filling the dermal papilla (Plate 4, Fig. 3). In some cases many granules remain outside the follicle, in the surrounding connective tissue, or clinging to the outside of the follicle (Plate 4, Fig. 3). At this stage the pigmented granules come into contact with the dendritic cells (Plate 4, Fig. 3) and the formation of hair pigment starts distal to the dendritic cells.

In the final stage, when the pigment in the hair is formed, the dermal papilla appears empty, except for a few remaining granules (Plate 4, Fig. 4) which disappear also after the hair is fully developed.

(iv) A great number of mast cells surround the arteries. There are no more mast cells around the veins than anywhere else in the surrounding tissue (see Plate 4, Fig. 3).

IV. Discussion

These observations, made on Naked and Crinkled mice, suggested that the pigmented mast cell granules outside and the granules inside the follicle were identical since all the intermediate stages of the "swallowing" of the granules by the papilla can be actually observed, as shown in Plates 2–4 and diagrammatically in Figure 1. Since in the full grown follicle all granules but a very few disappear from the dermal papilla (Plate 4. Fig. 4) and all the pigmented substance appears distal to the dendritic cells, we might suppose that these granules are used up in the development of the growing follicle.

If this is so, then in mice with reduced hair growth, such as Crinkled which has only 40 per cent. of the amount of hair in normal mice, a large number of granules should remain unused, provided the production of mast cells remains unimpaired.

All Crinkled mice of 6 months of age or above showed large numbers of pigmented granules and whole mast cells, turned black or brown depending on the coat colour of the animal, in the subdermal loose connective tissue sometimes stuck together in great lumps (Plate 1, Fig. 2). (Such residual granules can be observed in normal mice also, but only occasionally.)

It appears that mast cells, beside their function of producing heparin and histamine are also concerned in pigment formation and the growth of the hair follicle. The fact that the mast cell granules, either scattered or still inside the cell, turn to pigment in some cases suggests that the granules themselves are the precursors of the pigment in the follicle and that the mast cells contain a substance which can be converted into pigment. There is no pigment formation in the hair before the granules enter the papilla (Plates 2–4). Dendritic cells (Plate 4, Fig. 3) come in immediate contact with the granules packed inside the dermal papilla. Pigment formation distal to the dendritic cells does not start until after contact between mast cell granules and dendritic cells has been established and, as the pigment formation in the hair progresses, the dermal papilla becomes depleted of granules. A depleted papilla is shown in Plate 4, Fig. 4, containing only a few isolated granules.

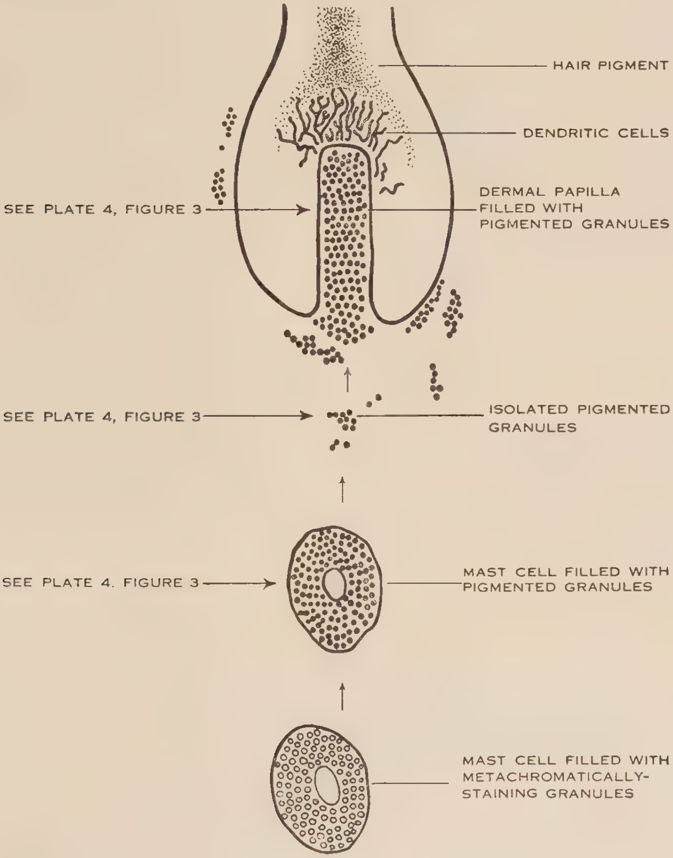

HAIR PIGMENT
DENDRITIC CELLS
SEE PLATE 4, FIGURE 3
DERMAL PAPILLA FILLED WITH PIGMENTED GRANULES
SEE PLATE 4, FIGURE 3
ISOLATED PIGMENTED GRANULES
SEE PLATE 4. FIGURE 3
MAST CELL FILLED WITH PIGMENTED GRANULES
MAST CELL FILLED WITH METACHROMATICALLY-STAINING GRANULES

Fig. 1.—Schematic illustration of the mast cell pigment relationship.

Medawar (1944, 1945, 1946*a*, 1946*b*, 1948*a*, 1948*b*) and Billingham and Medawar (1950) have shown that pigment formation is closely connected with the pigmentary dendritic cells, and that pigment spread seems to be caused "by a serially propagable transformation of non-pigmentary into pigmentary dendritic cells by a cytoplasmic ingredient of the latter which is capable of behaving 'infectively'."

The observations described in the present paper show that the granules in mast cells may be converted to pigment without coming into immediate contact with the dendritic cells.

Two important facts must be emphasized about this transformation: (i) the transformation occurs primarily below and round the hair follicles, and (ii) the colour of the transformed cells is always of the colour of the animal in question; black in black mice, brown in brown mice, light brown in diluted mice, very pale and just visible in chinchilla, an allele on the albino gene.

It must also be kept in mind that the transformation of mast cells into pigment outside the hair follicle is an abnormal process and may only indicate that under normal conditions the mast cells provide a "materia prima" which, after entering the hair follicle, is turned by dendritic cells into pigment, possibly after the dendritic cells have engulfed granules liberated by the mast cells, though this engulfing has not been observed. The intense metachromatic staining of the growing hair follicle which resembles the staining of mast cell granules seems to support such a view.

Two other possibilities may be considered: (iii) the mast cells and the dendritic cells independently follow different paths of pigment formation, and (iv) the mast cells receive pigment or pigmentary precursor from the follicular dendritic cells. At this stage, the author is unable to say without adequate experimental work what is the relationship of the dendritic cells to the pigmentary transformation of the mast cells; however, it seems to be sound, in face of the evidence, to discard these last two possibilities as unlikely.

V. ACKNOWLEDGMENTS

I wish to thank Dr. J. M. Rendel and Professor P. D. F. Murray for advice and criticism. I am also indebted to Mr. K. Adams for preparing the photographic plates.

VI. REFERENCES

BILLINGHAM, R. E., and MEDAWAR, P. B. (1950).—*Heredity* **4**: 2.
DRY, F. W. (1926).—*J. Genet.* **16**: 287.
EHRLICH, P. (1877).—*Arch. mikr. Anat.* **13**: 63.
GRÜNEBERG, H. (1952).—"Genetics of the Mouse." 2nd Ed. (Nijhoff: The Hague.)
HIRT, A. (1938).—*Verh. anat. Ges. Jena* **87**: 97-105.
HOLMGREN, H., and WILANDER, O. (1937).—*Z. mikr.-anat. Forsch.* **42**: 242.
JORPES, J. E., HOLMGREN, H., and WILANDER, O. (1937).—*Z. mikr.-anat. Forsch.* **42**: 279-301.
MEDAWAR, P. B. (1944).—*J. Anat., Lond.* **78**: 176.
MEDAWAR, P. B. (1945).—*J. Anat., Lond.* **79**: 157.
MEDAWAR, P. B. (1946*a*).—*Brit. J. Exp. Path.* **27**: 9.
MEDAWAR, P. B. (1946*b*).—*Brit. J. Exp. Path.* **27**: 15.
MEDAWAR, P. B. (1948*a*).—*Brit. J. Exp. Path.* **29**: 58.
MEDAWAR, P. B. (1948*b*).—*Quart. J. Micr. Sci.* **89**: 239.
RILEY, J. F. (1953*a*).—*J. Path. Bact.* **65**: 461, 471.
RILEY, J. F. (1953*b*).—*Science* **118**: 332.
RILEY, J. F. and WEST, G. B. (1953).—*J. Physiol.* **120**: 528.
ZAHL, P. A., and NOWAK, A. JR. (1949).—*Stain Tech.* **24**: 185.

EXPLANATION OF PLATES 1–4

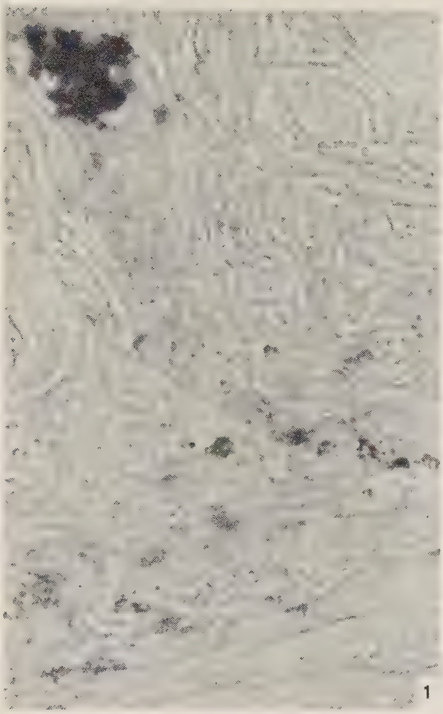
PLATE 1

Fig. 1.—Loose connective tissue between the dermal papilla packed with pigmented granules, and the subdermal loose connective tissue. Isolated mast cell granules scattered in the loose connective tissue. Unstained section (cf. Plate 2, Figs. 2, 3, and 4; Plate 3, Figs. 1, 2, and 3).

Fig. 2.—Lump of whole mast cells and their isolated granules, concentrated round a hair follicle, turned to pigment in the subdermal connective tissue of a Crinkled mouse. Unstained.

Fig. 3.—Mouse skin, whole mount, stained in methylene blue. *A*, artery; *V*, vein.

MAST CELLS AND HAIR GROWTH IN THE MOUSE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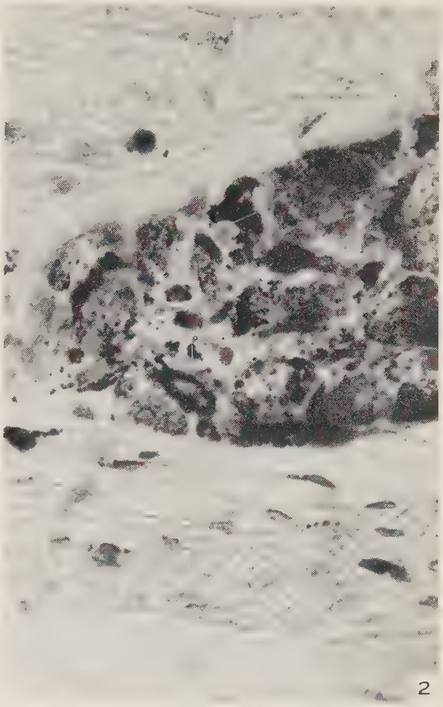
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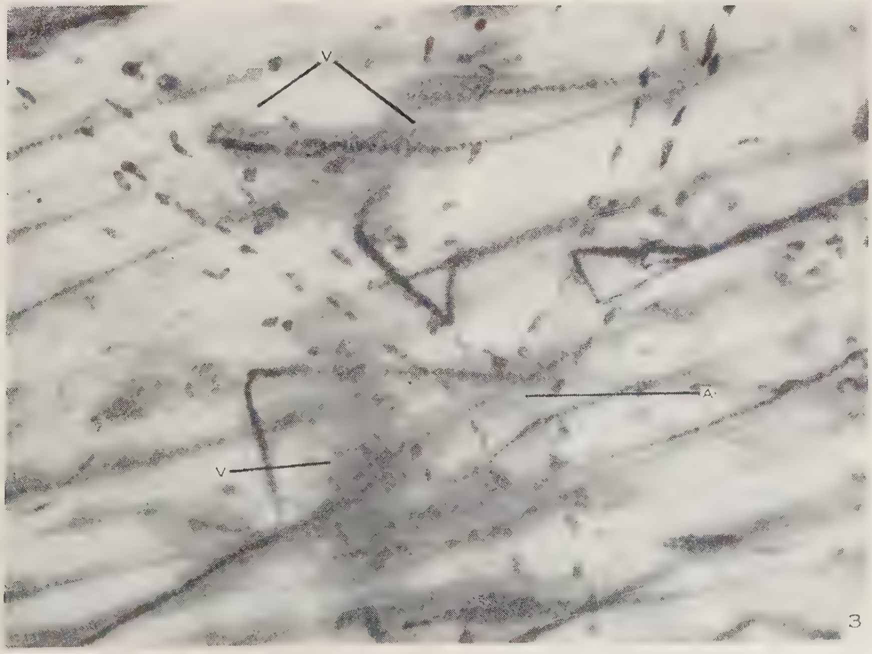

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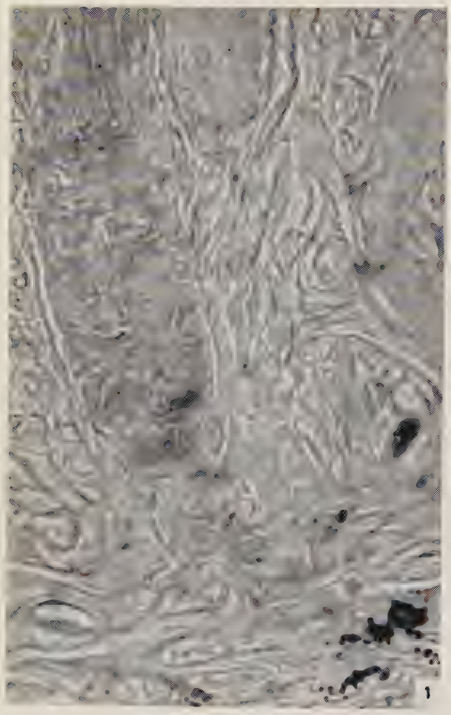

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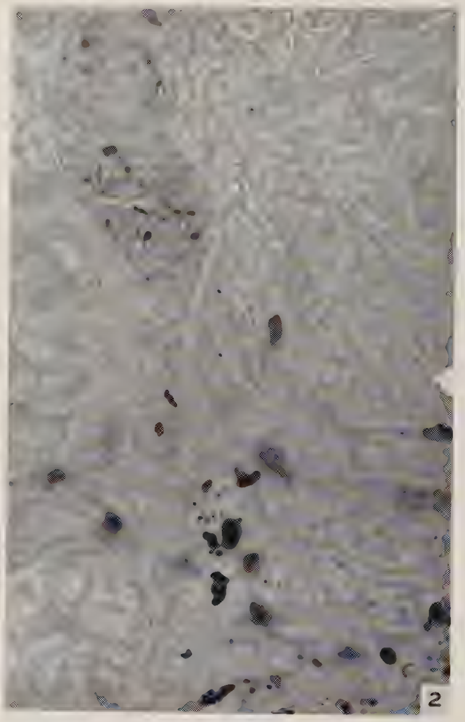

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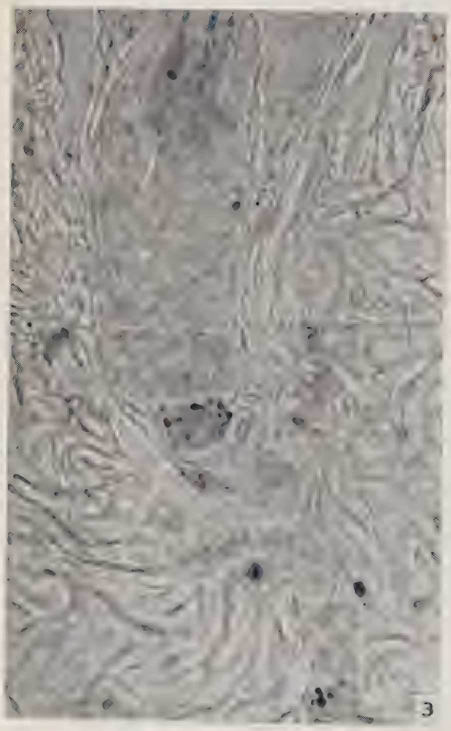

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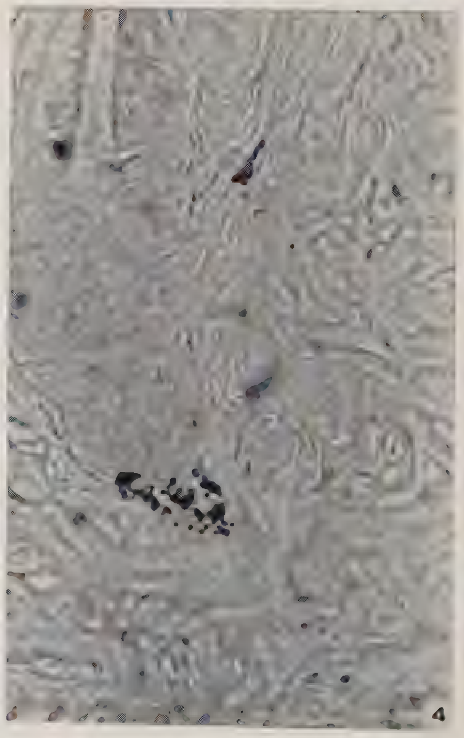

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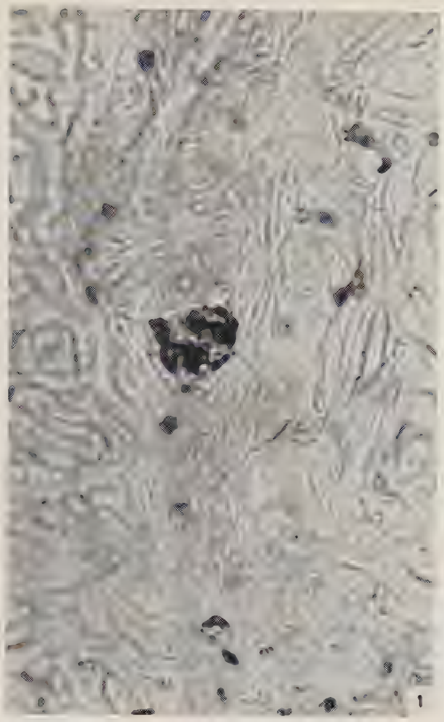

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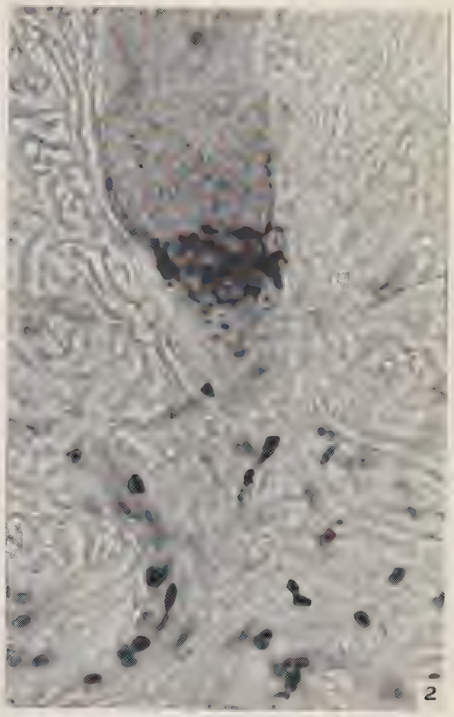

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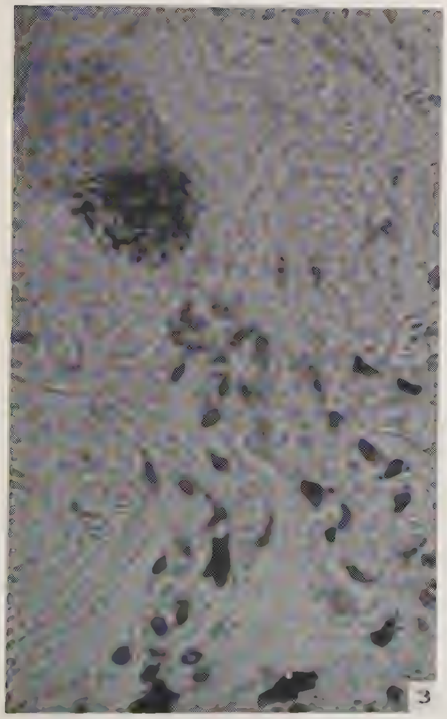

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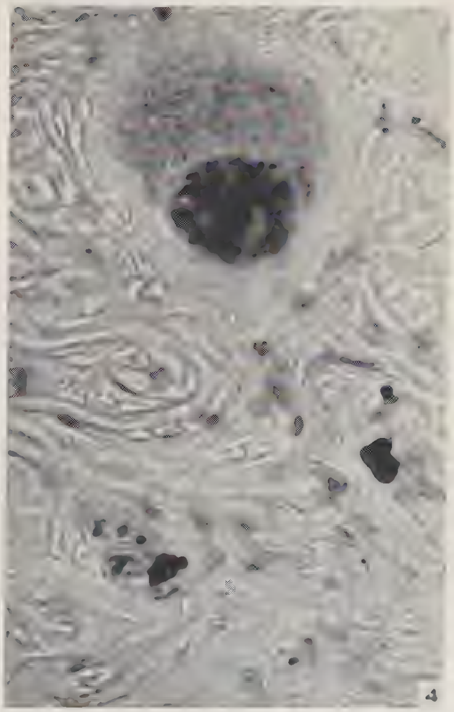

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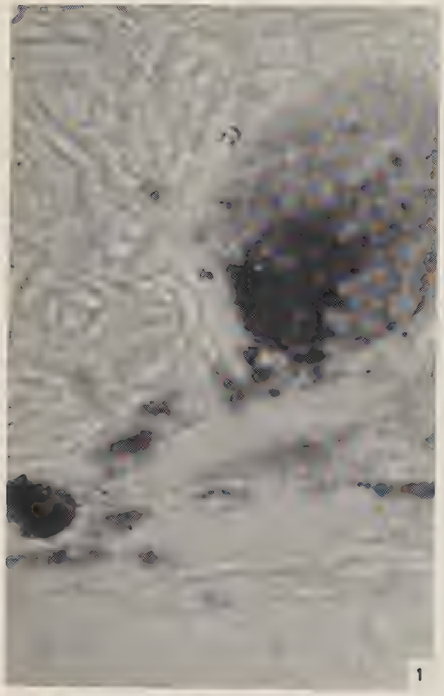

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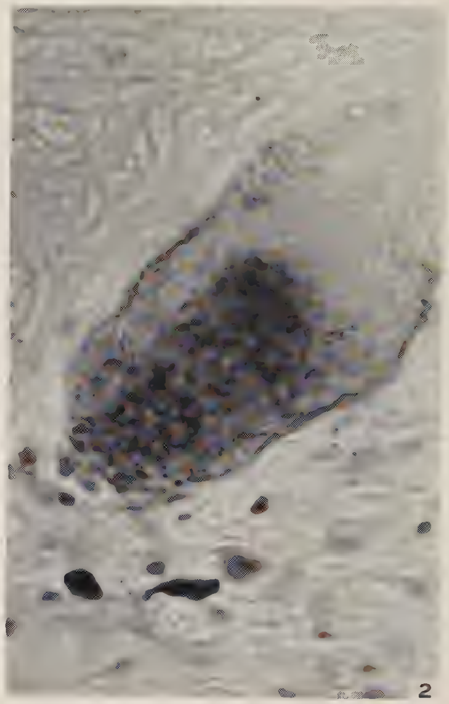

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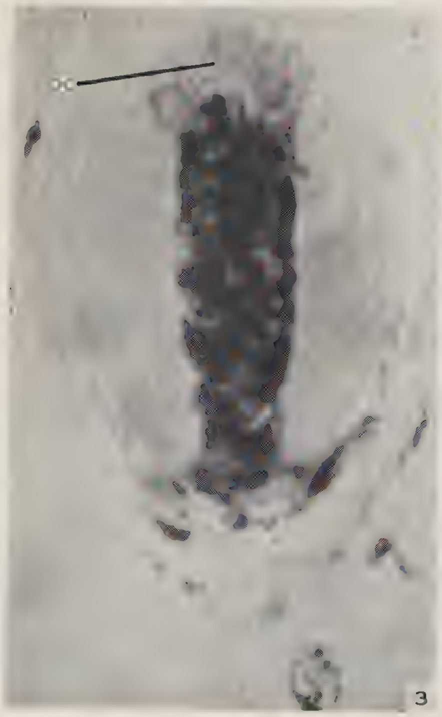

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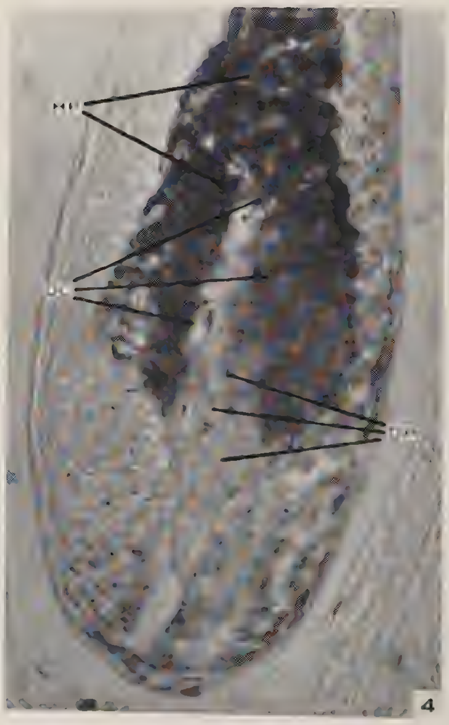

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PLATE 2

Fig. 1.—Dermal papilla in very early anagen, free from any pigment. Pigmented mast cells can be seen in the left lower corner of the figure. Unstained.

Fig. 2.—Isolated pigmented granules clinging to the outside of the dermal papilla of a growing hair follicle in early anagen. Note granule in the path of the growing follicle. Unstained.

Fig. 3.—Few pigmented granules can be seen inside the dermal papilla. Unstained.

Fig. 4.—Pigmented granules begin to concentrate in and round the dermal papilla. Unstained.

PLATE 3

Fig. 1.—Lump of pigmented granules becoming more compact. Note scattered mast cell granules in the loose connective tissue funnel below the follicle. Unstained.

Fig. 2.—Epidermal papilla begins to grow in an enveloping fashion round the lump of granules concentrated in and around the dermal papilla. Stained faintly in alcoholic thionin.

Fig. 3.—The same as for Plate 3, Figure 2, but in slightly more advanced stage. Note the loose connective tissue funnel below the follicle. Stained in alcoholic thionin.

Fig. 4.—The same as for Plate 3, Figure 3, but in more advanced stage. Stained in alcoholic thionin.

PLATE 4

Fig. 1.—Hair follicle in advanced growth stage, approaching subdermal loose connective tissue. The pigmented granule lump is almost swallowed by epidermal papilla. Pigmented mast cells are in front of the follicle, and some isolated granules scattered around the follicle. Stained in alcoholic thionin.

Fig. 2.—Similar stage to Plate 4, Figure 1, in side view. Stained in alcoholic thionin.

Fig. 3.—Dermal papilla filled completely with black pigmented granules. Scattered black granules are round the follicle in the surrounding connective tissue, one complete mast cell is filled with black granules in front of the follicle. Dendritic cells *(DC)* become visible on the distal end of the dermal papilla. Note lack of pigment distal from dendritic cells. Whole mount stained faintly with methylene blue.

Fig. 4.—Hair follicle in telogen stage. The pigmented granules, except for a very few, have disappeared from the dermal papilla. Hair pigment *(HP)* has appeared distal from the region occupied by the dendritic cells *(DC)*. *DP*, dermal papilla with isolated mast cell granules.

PROPERTIES OF WOOL ROOT PHOSPHATASES

By J. M. Gillespie*

[*Manuscript received April* 20, 1956]

Summary

Wool roots prepared by the wax sheet technique contain pyrophosphatase, phosphatase, and metaphosphatase. Of these the pyrophosphatase is the most active. It appears to carry an essential –SH group, is activated by Mg^{++} ions, and shows optimum activity at about pH 6·5. The phosphatase is not activated by metals nor inactivated by chelating agents or –SH inhibitors. Its optimum pH is not sharp but appears to be in the range 5–7. The metaphosphatase is activated by Mg^{++} and has an optimum pH of about 7.

I. Introduction

Pyrophosphatase, which is known to play a part in nucleotide metabolism (Kornberg 1950) is widely spread in nature. A comprehensive review of its distribution has been made by Roche (1950).

During the course of investigations concerning the enzymes in the wool root follicle, it was observed that pyrophosphatase and also certain other phosphatases were present. The activity of this group of enzymes appeared to be considerably greater than that of other enzyme systems in the wool root such as esterase, protease, and enzymes concerned with oxidation-reduction reactions. Ellis, Gillespie, and Lindley (1950) reported preliminary findings on the phosphatases. The present paper is concerned with the more detailed properties of the pyrophosphatase, phosphatase, and metaphosphatase of the wool root.

II. Materials and Methods

Sodium pyrophosphate was recrystallized twice from distilled water to free it completely from orthophosphate. Chemically pure sodium β-glycerophosphate was also used.

Pyrophosphatase activity was estimated by incubating 1·0 ml of enzyme solution or wool root suspension for 30 min at 37°C with 3·0 ml of a substrate buffered at pH 7·0. The final solution usually contained 0·5 mM sodium pyrophosphate, 75 mM sodium veronal, and 2 mM $MgCl_2$. The reaction was stopped by adding 2·0 ml of 20 per cent. trichloroacetic acid, the mixture filtered, and an aliquot taken for an inorganic phosphorus determination by the method of King (1932). Pyrophosphatase activity is recorded as the amount of orthophosphate liberated by 10 mg of wool roots in 30 min. The other phosphatases were estimated in a similar fashion using the appropriate substrate.

A mixed buffer containing 50 mM acetate and 50 mM borate, adjusted to the appropriate pH with acid or alkali, was used in experiments to assess the effect of pH on the activity of the enzymes.

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Wool roots were obtained from Merino cross type sheep, the method of preparation of roots being substantially that of Ellis (1948). Briefly, sheep skins were obtained within 30 min of removal from the animal, clipped to a wool length of about 3 mm, the wool embedded with a hot beeswax-rosin mixture, and the whole cooled to 10°C. The skin could then be stripped off, leaving the wool embedded in the wax with the root-ends protruding. The roots were removed with fine hair clippers and stored at −20°C or freeze-dried. In either case the phosphatases retained their activity for long periods.

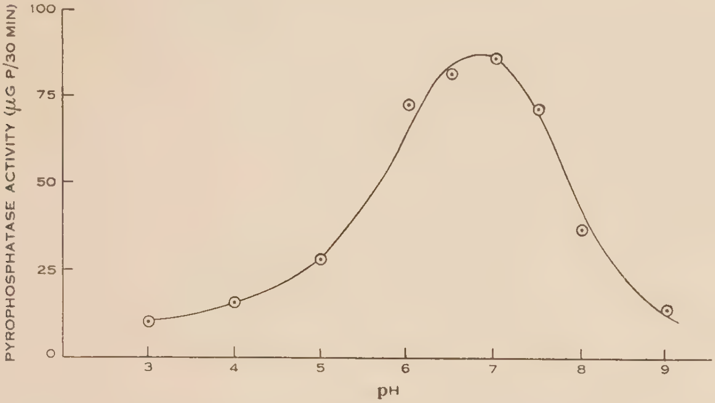

Fig. 1.—pH-Activity curve of wool root pyrophosphatase. System contained 50 mM acetate, 50 mM borate, 0·5 mM pyrophosphate, 2 mM Mg^{++}, and 10 mg wool roots; incubated for 30 min at 37°C.

Although the wax-sheet technique for preparing wool roots is very simple, it should be pointed out that it is very difficult to obtain reproducible yields of wool roots. For example one skin may yield 1 g of roots whilst several apparently similar ones may yield only 20 mg. This may be related to variations in the length of shaft in the skin, for it is suspected that a minimum length is needed for satisfactory cutting by the clippers.

In most enzyme tests a suitable amount of roots was added directly to the substrate. Simple extraction with water or salt solutions removed only small amounts of the pyrophosphatase, but better extraction was obtained after treatment with high intensity sound waves. The metaphosphatase and phosphatase were readily extracted with pH 4·5 buffer or with salt solution after autolysis.

III. Results

(a) *Pyrophosphatase*

(i) *Influence of pH on Activity.*—The pH-activity curve is shown in Figure 1. Maximum activity occurred at about pH 6·5.

(ii) *Activation by* Mg^{++}.—The influence of Mg^{++} on enzyme activity at a substrate concentration of 0·5 mM is shown in Figure 2. Maximum activity was attained at a Mg^{++} concentration over 0·001M. The enzyme was almost inactive in the absence of added Mg^{++}.

(iii) *Influence of Substrate Concentration on Activity.*—The relation between substrate concentration and reaction velocity at constant Mg^{++} concentration is shown in Figure 3. Increasing the pyrophosphate concentration increased the rate of hydrolysis up to a concentration of about 1 mM under standard conditions.

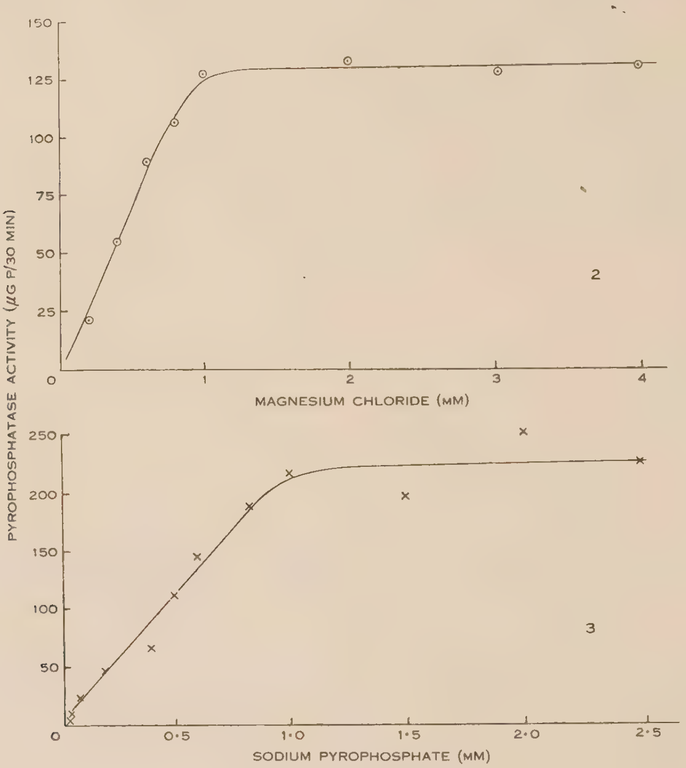

Fig. 2.—Activation of wool root pyrophosphatase by Mg^{++}. System also contained 75 mM veronal, 0·5 mM pyrophosphate, 10 mg wool roots; pH 7·0; incubated for 30 min at 37°C.

Fig. 3.—Influence of substrate concentration on the reaction rate of wool root pyrophosphatase. System also contained 75 mM veronal, 2 mM Mg^{++}, 10 mg wool roots; pH 7·0; incubated for 30 min at 37°C.

(iv) *Progress of Reaction.*—The relation between time and per cent. hydrolysis was almost linear up to 80 per cent. hydrolysis as shown in Figure 4.

(v) *Inhibitors.*—Table 1 lists the effect of some inhibitors on the wool root enzyme. The inactivation by oxygen could be completely reversed by the addition of cysteine, and a similar loss of activity on long storage at room temperature could also be reversed by reduction.

(vi) *Effect of Dialysis.*—After dialysis against distilled water, wool roots and their extracts showed no pyrophosphatase activity, but activity could be completely restored by the addition of cysteine and Mg^{++} (see Table 2).

(vii) *Other Sources of Pyrophosphatase.*—Qualitative tests showed that bovine horn pre-keratin and the roots of rabbit and human hair also contained pyrophosphatase.

(*b*) *Metaphosphatase*

(i) *Optimum pH.*—It can be seen from Figure 5 that the highest activity is obtained near pH 7, but that the enzyme retains considerable activity over a wide pH range.

(ii) *Activation.*—The enzyme required Mg^{++} ions for its activity and was not activated by Ca^{++}, Zn^{++}, Mn^{++}, or Co^{++} ions.

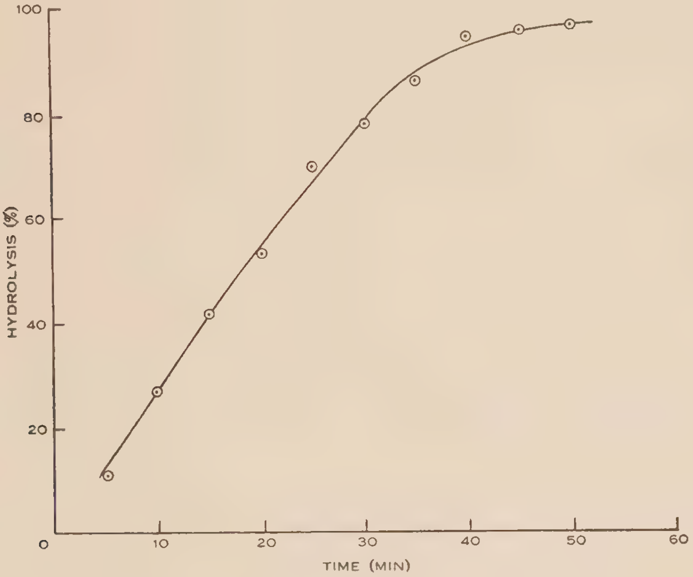

Fig. 4.—Influence of time on the hydrolysis of pyrophosphate by wool root pyrophosphatase. System contained 20 mM Mg^{++}, 75 mM veronal, 0·5 mM pyrophosphate, 10 mg wool roots; pH 7·0; incubated for 30 min at 37°C.

(*c*) *Phosphatase*

(i) *pH-Activity Curve.*—The relation between pH and enzyme activity is shown in Figure 6. The curve is rather spread out, but the maximum activity seems to be in the pH region 5–7.

(ii) *Activation.*—This enzyme appeared to be fully active in the isolated wool roots, and no increase in activity was observed after adding Ca^{++}, Mg^{++}, Zn^{++}, Mn^{++}, or Co^{++} ions. It was not inactivated by 100 mM concentrations of fluoride, 8-hydroxyquinoline, or iodoacetic acid.

(iii) *Tests Against Other Substrates.*—Table 3 shows the results obtained using a number of organic phosphorus compounds as substrates.

IV. DISCUSSION

The present work suggests that the pyrophosphatase of the wool root resembles those of yeast and erythrocytes, especially in respect of the action of inhibitors and activators. The optimum pH near 6·5 is close to that reported for the pyrophosphatase of yeast (pH 7) by Bailey and Webb (1944) and for those of many animal tissues, but it is lower than the optimum value of pH 8·3 for enzymes from insect muscle (Gilmour and Calaby 1953) and from the fire-fly (McElroy, Coulombre, and Hays 1951).

TABLE 1

EFFECT OF SOME INHIBITORS ON THE ACTIVITY OF WOOL ROOT PYROPHOSPHATASE

Wool root suspension incubated with inhibitors in the presence of Mg^{++} for 30 min at 37°C before addition of buffer substrate. Final conditions: 2 mM Mg^{++}, 0·5 mM pyrophosphate, pH 7. Complete system incubated at 37°C for further 30 min

Inhibitor	Concn. (mM)	Inhibition (%)
p-Chloromercuribenzoate	10	100
Iodoacetate	50	95
o-Iodosobenzoate	10	100
Fluoride	0·1	100
8-Hydroxyquinoline	0·1	100
Citrate	0·1	85
Ca^{++}	10	100
Cu^{++}	1	100
Formalin	10	100
Oxygen (bubbled)	—	100

TABLE 2

EFFECT OF DIALYSIS ON THE ACTIVITY OF WOOL ROOT PYROPHOSPHATASE

Wool root extract dialysed against running tap water for 24 hr and then against frequently-changed distilled water for 24 hr at 2°C. Final conditions: 0·5 mM pyrophosphate, pH 7. System incubated at 37°C for 30 min

Preparation	Activity (μg P/30 min)
Wool root extract (control)	10
Wool root extract + 20 mM cysteine	24
Wool root extract + 10 mM Mg^{++}	60
Wool root extract + 20 mM cysteine + 10 mM Mg^{++}	66
Dialysed wool root extract	0
Dialysed wool root extract + 20 mM cysteine	10
Dialysed wool root extract + 10 mM Mg^{++}	0
Dialysed wool root extract + 20 mM cysteine + 10 mM Mg^{++}	70

It has been shown that the wool root pyrophosphatase requires Mg^{++} for activity and the optimum concentration corresponds with a molar ratio of Mg^{++} to substrate of about 2:1 which is the same as that reported for the pyrophosphatases of muscle and liver (Lohman 1933), erythrocytes (Bloch-Frankenthal 1954), and insect muscle (Gilmour and Calaby 1953). The significance of this has been recently discussed by Bloch-Frankenthal (1954).

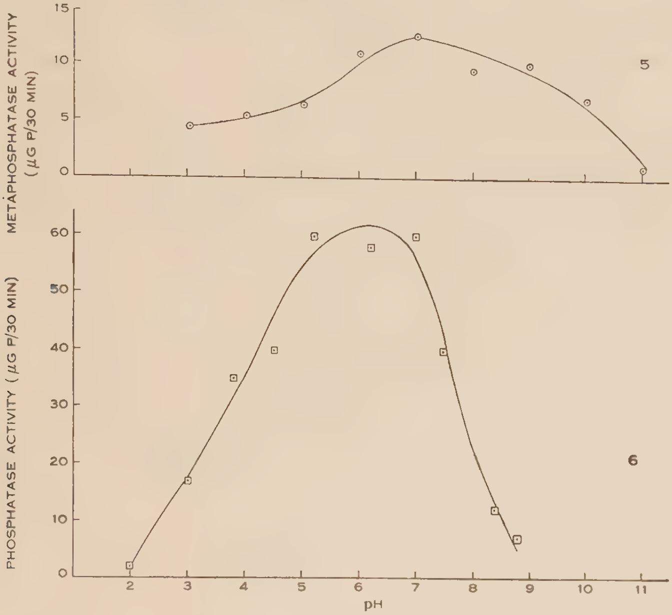

Fig. 5.—pH-Activity curve of wool root metaphosphatase. System contained 50 mM acetate, 50 mM borate, 1·0 mM metaphosphate, 50 mg wool roots; incubated for 30 min at 37°C.

Fig. 6.—pH-Activity curve of wool root phosphatase. System contained 50 mM acetate, 50 mM borate, 1·0 mM β-glycerophosphate, 50 mg wool roots; incubated for 30 min at 37°C.

Table 3

PHOSPHATASE HYDROLYSIS OF SOME ORGANIC PHOSPHORUS SUBSTRATES

Final solution contained 20 mg wool roots, 1·0 mM substrate buffered to pH 6, 20 mM acetate. System incubated at 37°C for 30 min

Substrate	Activity (μg P/30 min)
α-Glycerophosphate	5
β-Glycerophosphate	20
Fructose-1, 6-diphosphate	6
Adenylic acid	7
Disodium phenylphosphate	22·5
Phytin	0

Wool roots do not appear to contain large amounts of the enzyme in comparison with the crude enzyme preparations reported on by other workers. It is difficult to make an exact comparison, but, using the figures of Heppel and Hilmoe (1951) for their best method of extraction, dried yeast yielded something over three times the activity given by an equal weight of dried wool roots.

Since all the reagents reacting with –SH groups reduce the activity it seems that this enzyme has an essential –SH group. The concentration required for inactivation was often higher than that reported by other workers and this may have been due to reaction of the reagents with –SH associated with the wool root protein.

Inhibition by formaldehyde may indicate that intact amino groups in the enzyme are also essential but this is not certain since this reagent also reacts with –SH groups.

Good reproducibility was very difficult to obtain because of the varying amounts of shaft and root in different preparations. In a typical preparation, less than 30 per cent. of the protein was easily water soluble and very little enzyme was extracted with it. A variation of up to 50 per cent. in pyrophosphatase activity was observed between different preparations, and it was not possible therefore to calculate the reaction rates.

V. REFERENCES

BAILEY, K., and WEBB, E. C. (1944).—*Biochem. J.* **38**: 394.
BLOCH-FRANKENTHAL, L. (1954).—*Biochem. J.* **57**: 87.
ELLIS, W. J. (1948).—*Nature* **162**: 957.
ELLIS, W. J., GILLESPIE, J. M., and LINDLEY, H. (1950).—*Nature* **165**: 545.
GILMOUR, D., and CALABY, J. H. (1953).—*Enzymologia* **16**: 34.
HEPPEL, L. A., and HILMOE, R. J. (1951).—*J. Biol. Chem.* **192**: 87.
KING, E. J. (1932).—*Biochem. J.* **26**: 292.
KORNBERG, A. (1950).—*J. Biol. Chem.* **182**: 779.
LOHMANN, K. (1933).—*Biochem. Z.* **262**: 137.
MCELROY, W. D., COULOMBRE, J., and HAYS, R. (1951).—*Arch. Biochem.* **32**: 207.
ROCHE, J. (1950).—"The Enzymes." Vol. 1. Pt. 1. p. 493. (Ed. J. B. Sumner and K. Myrbäck.) (Academic Press Inc.: New York.)

FERTILITY IN RABBITS RECOVERING FROM MYXOMATOSIS

By W. R. Sobey* and K. Turnbull†

[*Manuscript received February* 15, 1956]

Summary

The fertility of 20 bucks and 13 does which recovered from an infection of myxoma virus, strain KM.13, under laboratory conditions was examined. Six months after infection, only 50 per cent. of the bucks were found to be fertile and many of these were not fully fertile. Three females were found to be infertile, of which one could be bred from by using artificial insemination, but the fecundity of the remainder was unimpaired. The reduced fertility observed under laboratory conditions is not necessarily a reflection of field conditions where most of the infertile animals may not survive.

I. Introduction

In the course of a selection programme for a line of rabbits resistant to the KM.13 strain of myxoma virus, the fertility of all animals recovering from the disease was examined as a pre-requisite to selection. This paper deals with the observed fertility of 20 bucks and 13 does which have recovered in this Laboratory during the past 12 months.

II. Materials and Methods

The rabbit colony was housed in two, large, well-ventilated, unheated rooms. The breeding stock was genetically heterogenous, including both domestic and wild rabbits. Animals forming part of the selection programme were infected at between 4 and 5 months of age by inoculation with about 10 ID_{50} strain KM.13. After infection, animals were observed daily for a period of 60 days, after which the surviving animals were examined weekly with respect to fertility. It was found possible to classify the severity of the symptoms shown by an infected rabbit into three main classes, based on the general physical condition of the rabbit and the time after infection at which it can safely be judged to have recovered. They are as follows:

"+"—A mild attack with no marked loss of condition, and a marked localization of lesions wherever they erupt at the skin surface. Animals of this group can be predicted as recoveries about 24 days after infection.

"++"—More severe attack, loss of condition, and no localization of lesions. Prediction of recovery cannot be made before 34 days after infection.

"+++"—Severe attack, generalization of the disease with marked loss of condition, and prediction of recovery impossible before 44 days after infection.

Having been classified as recoveries, rabbits were examined periodically for sexual interest. Does were presented to a healthy buck and their acceptance or refusal of the buck during a period of about 15 min used as an index of sexual interest. Recovered bucks were presented with a healthy female and their interest judged on

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whether or not they will mount during a period of about 15 min. As soon as bucks displayed sexual interest, they were introduced to an artificial vagina, similar to the one described by White (1955), used in conjunction with a teaser doe. The ejaculate, when present, was examined microscopically after dilution in 0·9 per cent. saline. Bucks were graded into categories 1–6 (see Table 1).

Table 1

GRADATION OF BUCKS INTO DIFFERENT CATEGORIES AFTER SPERM EXAMINATION

Category	Sexual Interest	Ejaculate	Remarks
1	–	–	
2	+	–	
3	+	+	No sperm
4	+	+	Sperm present, non-motile, *infertile*
5	+	+	Sperm present, sparse, motile, *intermittently fertile*
6	+	+	Plentiful motile sperm, *fully fertile*

Table 2

COMPARISON OF MEAN LITTER SIZE FOR BUCKS FALLING INTO CATEGORIES 5 AND 6 AT DIFFERENT TIMES

Rabbit No.	Category 5			Category 6		
	Mean Litter Size	No. of Matings Without Conception	Total No. of Matings	Mean Litter Size	No. of Matings Without Conception	Total No. of Matings
131	2·5	4	6	7	0	3
145	3·0	2	5	5·5	0	2
189	3·0	0	1	6·5	0	4
257	3·5	1	3	6·6	0	3
681	3·25	0	4	5·8	0	9
695	3·5	2	4	7·0	0	8
783	—	—	—	6·2	0	5
930	3·75	1	5	7·1	0	6
155A	1·0	0	1	6·9	0	8

III. Results

The difference in relative fertility between category 5 and category 6 bucks is contrasted in Table 2 where the fertility of the same bucks falling into either of the two categories at different times is compared. The difference between mean litter size, ignoring the matings without conception, is significant ($P < 0 \cdot 02$). The mean litter size for bucks in category 6 is $6 \cdot 58 \pm 0 \cdot 19$, and mean litter size for those in category 5 is $3 \cdot 16 \pm 0 \cdot 21$, $t_7 = 3 \cdot 22$.

Using the scale in Table 1, the data collected for bucks are set out in Table 3. The animals to the right of the table had fewer semen collections recorded as they were more recent recoveries. It is apparent that not all bucks become fertile even 6 months after recovering, some showing no sexual interest at all. Of those which did become fertile, only two appeared consistently fully fertile, of which one, No. 155A, had been examined for a period of 6 months, and one, No. 783, for a period of 12 months.

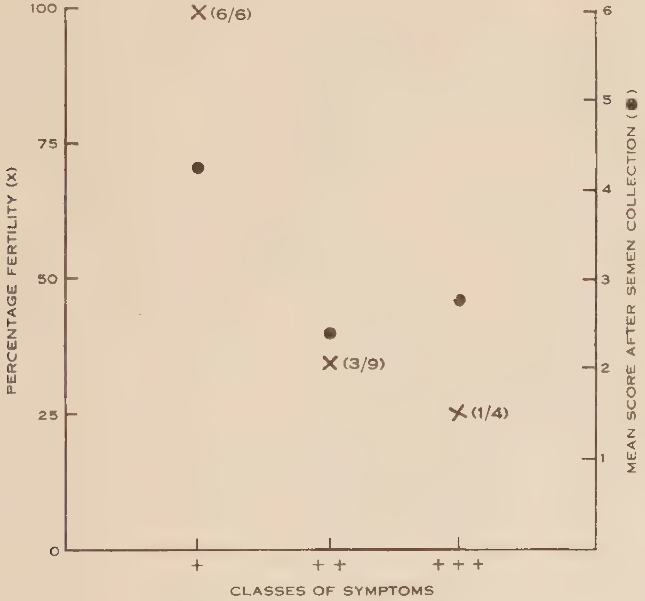

Fig. 1.—Change in percentage fertility (×) and mean score (●) in relation to the severity of the symptoms. Rabbit No. 436A (see Table 3) has been excluded since it has not been tested over a period of 6 months.

The severity of the disease in bucks which eventually recovered appeared to have a marked influence on fertility. At 6 months after infection, the number of fertile bucks in each of the three classes of symptoms were plotted in Figure 1 as percentages against these classes. Both categories 5 and 6 (Table 3) are classified as fertile. The mean scores of each class, where score is the category number as in Table 1, are also given. The data are too limited to differentiate between "++" and "+++" classes both of which display markedly reduced fertility, but the difference between these two classes and the "+" class, which is almost fully fertile, is quite clear.

The fertility of does recovering from the disease was much less variable than bucks. Of the 13 animals under observation, shown in Table 4, two were infertile, one being tested over a period of 12 months and one over 6 months. One doe, No. 607, refused the buck during the 8 months under test but proved fertile when artificially inseminated. As routine artificial insemination involves the injection of 100 units

TABLE 3

CHANGES IN THE FERTILITY OF BUCKS UP TO 12 MONTHS AFTER INFECTION IN RELATION TO THE SEVERITY OF THE DISEASE

Numbers refer to the categories as given in Table 1

Rabbit No.		131	145	147	188	189	257	588	605	681	748	783	930	168A	173A	235A	151A	155A	158A	357A	436A
Severity of reaction		++	+++	+++	++	++	+++	+++	++	+	++	+	+	++	++	+	++	+	++	+	++
Months	Weeks (approx.)	Semen Tests after Infection																			
2																					
3	1					1	1													3	1
	2	3				3	5													5	2
	3	3			1	3						6	5	1	1	1	1	1	1		
	4	3			1	3		1	1	6	1	5	5	3	1	1	1	1	1		
4	1				1	3				5			5	3	1	1	1	1	1		
	2						5							3	1	1	2	6	1		
	3	3				6				5			5	3	1	1	2	6	1		
	4	3				6		3		5	1			3	2	2	3	6	1		
5	1		1	2		6				5	2		5	3	3	2	3	6	1		
	2	3	3	1		6	5			5	3					2		6			
	3	3	3	1	1	5												6			
	4	3	3		1			3		5		5		3	3		3	6			
6	1					5	3	3	6	5		5	5								
	2	3	3		1	3	6		6	6	3		5								
	3	6	3	2			5			5	2		6	3	3	2	3	6	1		
	4	6	3				6		5	5				3	3	5	2	6	1		
7	1	6				3	6				2										
	2					3	5														
	3	3																			
	4	3					6														

TABLE 3 (*Continued*)

Rabbit No.		131	145	147	188	189	257	588	605	681	748	783	930	168A	173A	235A	151A	155A	158A	357A	436A
Severity of reaction		++	+++	+++	++	++	+++	+++	++	+	++	+	+	++	++	+	++	+	++	+	++
Months	Weeks (approx.)	Semen Tests after Infection																			
8	1	6	5			3	5														
	2		5	2		3															
	3																				
	4	3			1		3					6	5								
9	1	3	6	1		3						6	6								
	2			1	1																
	3	3				3	3														
	4				1																
10	1	6	6	1								6									
	2																				
	3																				
	4	3																			
11	1	6	6					3													
	2		6																		
	3						3					6									
	4	3	5																		
12	1																				
	2																				
	3																				
	4	5										6									
Mean score at 6 months		3·5	2·7	1·5	1·0	4·2	4·5	2·5	4·5	5·2	2·0	5·2	5·1	2·8	1·9	1·8	2·1	4·8	1·0	4·0	1·5

of gonadotrophin to ensure ovulation, the natural fertility of this doe is in some doubt. All the other does were fully fertile by 5 months, accepting a buck normally. The fecundity of the recovered does which breed did not appear to be affected. Their mean litter size of 5·71 ± 0·49 was not significantly different from that of control animals—6·14 ± 0·26, $t_{95} = 0{\cdot}754$.

TABLE 4

FERTILITY OF DOES UP TO 12 MONTHS AFTER INFECTION IN RELATION TO THE SEVERITY OF THE DISEASE

Doe No.		445	608	157	437	127	134	136	128	191	715	923†	132‡	607
Severity of reaction		+	+	+	+	++	++	++	++	++	++	++	+++	+++
Months	Weeks (approx.)	Time after Infection to Birth of First Litter (denoted by asterisk)												
2														
	1				*									
	2													
	3	*												
3	4													
	1													
	2													
	3			*			*	*			*			*§
4	4													
	1		*			*			*	*				
	2													
	3													
5	4													

† Doe No. 923 infertile after 6 months under test.

‡ Doe No. 132 infertile after 12 months under test.

§ Litter born only after artificial insemination.

IV. DISCUSSION

Fenner *et al.* (1953) and Marshall *et al.* (1955) found no effects on the fecundity of females in large samples of field recoveries, and to this extent the present data are in agreement. Unfortunately their sampling methods would not disclose completely infertile females, and no estimate of their occurrence under field conditions can be made. It is conceivable that under the more rigorous conditions prevailing in the field, only the "+" and possibly some of the "++" classes would survive. Under such circumstances few infertile animals would be encountered, although some of the bucks would probably be only intermittently fertile. This would tend to work against the spread of genetic resistance where recovered bucks are in competition with bucks that have never been infected, but will increase the selection for resistance in areas where infection is complete.

V. References

Fenner, F., Marshall, I. D., and Woodroofe, Gwendolyn, M. (1953).—*J. Hyg.* **51**: 15.
Marshall, I. D., Dyce, A. L., Poole, W. E., and Fenner, F. (1955).—*J. Hyg.* **53**: 12.
White, I. G. (1955).—*Aust. J. Exp. Biol. Med. Sci.* **33**: 367.

CONCEPT OF GENERAL AND SPECIFIC COMBINING ABILITY IN RELATION TO DIALLEL CROSSING SYSTEMS

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Summary

A detailed examination of the concept of combining ability in relation to diallel crossing systems is made. Eight different analyses are presented. These result from a consideration of four different diallel crossing systems together with two alternative assumptions with regard to the sampling nature of the experimental material. A numerical example is given.

I. Introduction

The concept of combining ability is becoming increasingly important in plant and animal breeding. It is especially useful in connection with "testing" procedures, in which it is desired to study and compare the performances of lines in hybrid combination. In this paper we are concerned with the definitions of general and specific combining ability when the testing procedure utilizes a diallel crossing system. It was with such a system that the terms general and specific combining ability were originally defined by Sprague and Tatum (1942). They defined the terms as follows: "The term 'general combining ability' is used to designate the average performance of a line in hybrid combination. . . . The term 'specific combining ability' is used to designate those cases in which certain combinations do relatively better or worse than would be expected on the basis of the average performance of the lines involved."

By a diallel crossing system is meant one in which a set of p inbred lines is chosen and crosses among these lines are made. This procedure gives rise to a maximum of p^2 combinations. Data from such combinations can be most conveniently set out in a $p \times p$ table in which x_{ii} represents the mean value for the ith inbred, x_{ij} the mean value for the F_1 resulting from crossing the ith and jth inbreds, and x_{ji} represents its reciprocal. Thus the p^2 combinations can be divided into three groups: (1) the p parental lines themselves, (2) one set of $\frac{1}{2}p(p-1)$ F_1's, and (3) the set of $\frac{1}{2}p(p-1)$ reciprocal F_1's.

Even though this discussion is restricted to diallel crosses, we must still consider possible variations in the diallel crossing system itself and in the assumptions concerning the sampling nature of the experimental material.

Diallel crossing techniques may vary depending upon whether or not the parental inbreds or the reciprocal F_1's are included or both. With this as a basis for classification there are four possible experimental methods: (1) parents, one set of F_1's and reciprocal F_1's are included (all p^2 combinations); (2) parents and one set of F_1's are included but reciprocal F_1's are not ($\frac{1}{2}p(p+1)$ combinations); (3) one set of F_1's and reciprocals are included but not the parents ($p(p-1)$ combinations); and (4) one set of F_1's but neither parents nor reciprocal F_1's is included ($\frac{1}{2}p(p-1)$ combinations). Each method necessitates a different form of analysis.

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With regard to the sampling assumptions it is necessary to distinguish between (1) the situations in which the parental lines simply or the experimental material as a whole are assumed to be a random sample from some population about which inferences are to be made, and (2) the situations in which the lines are deliberately chosen and cannot be regarded as a random sample from any population. This second assumption can be expressed somewhat differently by stating that the experimental material constitutes the entire population about which valid inferences can be made.

These two different assumptions give rise to different estimation problems and different tests of hypotheses regarding combining ability effects.

The purpose of this paper is to bring together the eight different analyses arising from the different assumptions and diallel methods, to indicate the situations in which each might be used, and finally, to give a detailed numerical example. It is hoped that this will contribute to the usefulness of the ever-increasing application of the concept of combining ability in conjunction with diallel crosses.

II. PRESENTATION OF ANALYSES

(*a*) *Introduction*

For each of the four experimental methods we shall consider separately the two analyses arising from the different assumptions regarding the sampling nature of the experimental material. However, we must first consider the experimental design which is employed in obtaining the experimental data.

We shall restrict our analyses to conform to only one design, namely the randomized-blocks design. We choose this design primarily because of its common usage and its applicability to this type of study. The combining ability analyses can easily be used with various other designs.

In the randomized-blocks design we assume that there are a varieties (i.e. the genotypes determined by the diallel crossing method), each of which is assigned at random to each of b blocks, and that there are c individuals in each of the ab plots. Thus the mathematical model for the $ijkl$th observation is assumed to be

$$x_{ijkl} = u+v_{ij}+b_k+(bv)_{ijk}+e_{ijkl},$$

where u is the population mean effect, v_{ij} is the effect for the ijth genotype, b_k is the kth block effect, $(bv)_{ijk}$ is the interaction between the ijth genotype and the kth block, and e_{ijkl} is the environmental effect peculiar to the $ijkl$th individual. A double subscript notation is used for the variety (i.e. genotypic) effect because it is desired to denote the genotypic means in the combining ability analyses as x_{ij}, where x_{ii} is the mean for the ith parent, and x_{ij} is the mean for the F_1 resulting from crossing the ith and jth parents. In the combining ability analyses, the variety effects are considered in terms of general and specific combining ability effects, such that

$$v_{ij} = g_i+g_j+s_{ij}$$

for those diallel crossing methods in which reciprocal F_1's are not included, and

$$v_{ij} = g_i+g_j+s_{ij}+r_{ij}$$

for methods in which reciprocal F_1's are included. In these equations g_i is the general combining ability (denoted as g.c.a.) effect of the ith parent, s_{ij} is the specific combining ability (denoted as s.c.a.) effect for the cross between the *i*th and *j*th parents, and r_{ij} is the reciprocal effect involving the *i*th and *j*th parents.

In this study we wish to present analyses for the two assumptions in which the genotypes are assumed to be (1) a random sample from a population, and (2) a chosen or fixed set. In the first situation the genotypic effects are considered random variables and in the second they are considered constants. We must now consider how these assumptions are to be integrated with the more general set of assumptions which are made with regard to the elements in the mathematical model for the randomized-block design.

There are four sets of assumptions which can be considered with regard to the variety (genotypic) and block effects. These are (1) the variety and block effects are constants, (2) the variety effects are random variables and the block effects are constants, (3) the variety effects are constants, and the block effects are random variables, and (4) the variety and block effects are both random variables.

The first set of assumptions leads to a model in which all effects except the error are regarded as constants. The last set of assumptions leads to a model in which all effects except u are random variables. These two classes of models have been designated as models I and II respectively by Eisenhart (1947). The second and third sets of assumptions lead to mixed models which we designate as mixed A and mixed B respectively. Table 1 gives the analysis of variance for the randomized-block design together with the expectations of mean squares for each of the four sets of assumptions.

In the analyses to follow, we shall consider only those two sets of assumptions characterized by models I and II. However, the mixed models can be used in all cases, and their application will be briefly described later.

It is clear that the objectives of the analyses and the analyses themselves are different for the two basic assumptions regarding the experimental material.

In model I the experimental material is to be regarded as the population about which inferences are to be made. The objectives are to compare combining abilities of the parents when the parents themselves are used as testers, and to identify the higher yielding combinations. Thus we are particularly interested in estimating combining ability effects and computing appropriate standard errors for differences between effects. For testing procedures it is necessary to assume only that the e_{ijkl} are normally and independently distributed with mean zero and variance σ_e^2.

In model II the assumption is that we are dealing with random samples from some parent population, and the inferences are not to be made about the individual lines in the sample but about the parameters in the parent population. In particular, we are interested in estimating the genetic and environmental components of the complex population variance. To do this we assume that the effects in the model (except u) are normally and independently distributed with means zero and variances σ_θ^2, where $\theta = b, g, s$, or r. The variance component estimates are then obtained for any given diallel crossing method by equating the observed to the expected mean squares in the appropriate analysis of variance. The standard errors for the variance component estimates are calculated from the variances of the appropriate mean squares. The procedure will be indicated for each experimental method.

TABLE I

RANDOMIZED-BLOCKS ANALYSIS OF VARIANCE GIVING EXPECTATIONS OF MEAN SQUARES FOR THE ASSUMPTIONS OF MODELS I, II, MIXED A, AND MIXED B

Source	D.F.	Mean Square*	Expectations of Mean Squares			
			Model I	Model II	Mixed A	Mixed B
Varieties	$a-1$	M_v	$\sigma_e^2+bc\phi(v)$	$\sigma_e^2+c\sigma_{bv}^2+bc\sigma_v^2$	$\sigma_e^2+bc\sigma_v^2$	$\sigma_e^2+c\phi_2(bv)+bc\phi(v)$
Blocks	$b-1$	M_b	$\sigma_e^2+ac\phi(b)$	$\sigma_e^2+c\sigma_{bv}^2+ac\sigma_b^2$	$\sigma_e^2+c\phi_3(bv)+ac\phi(b)$	$\sigma_e^2+ac\sigma_b^2$
Varieties × blocks	$(a-1)(b-1)$	M_{bv}	$\sigma_e^2+c\phi_1(bv)$	$\sigma_e^2+c\sigma_{bv}^2$	$\sigma_e^2+c\phi_3(bv)$	$\sigma_e^2+c\phi_2(bv)$
Error	$ab(c-1)$	M_e	σ_e^2	σ_e^2	σ_e^2	σ_e^2

* Where

$$\phi(v) = \frac{1}{a-1}\Sigma v_i^2; \qquad \phi_1(bv) = \frac{1}{(a-1)(b-1)}\sum_{i<j}\sum_k\Sigma(bv)_{ijk}^2;$$

$$\phi(b) = \frac{1}{b-1}\sum_k b_k^2; \qquad \phi_2(bv) = \frac{1}{a-1}\sum_{i<j}\Sigma(bv)_{ijk}^2;$$

$$\phi_3(bv) = \frac{1}{b-1}\sum_k(bv)_{ijk}^2.$$

It should be emphasized that the proper interpretation of the combining ability effects and variance depends on the particular diallel method, the assumptions regarding the experimental material, and the conditions imposed on the combining ability effects. For example, when model I is used the equations for estimating the combining ability effects vary from one diallel method to another. These estimators yield unbiased estimates of the combining ability effects only when the specified constraints are imposed on the elements. These constraints vary from one method to another. Finally, valid inferences can be made only about the particular experimental material used. When model II is used, the kinds of inferences which can validly be made will depend on the particular diallel crossing method employed and on the nature of the population from which the lines were drawn.

In the following analyses we are concerned with equal subclass numbers. However, in certain problems, particularly those dealing with animals, the investigator is forced to consider unequal subclass numbers. Henderson (1948, 1952) has provided solutions to problems of estimation and tests of hypotheses for method 3, model II, for this type of data.

In order to clarify the notation used for the four diallel methods, an illustration is given for the case of $p = 3$. Since in all of the analyses only genotypic means are involved we shall simplify the usual notation for the mean of the ijth genotype from $\bar{x}_{ij..}$ to x_{ij}. The 3×3 table of mean observations may be set out as follows:

$$\begin{array}{ccc} x_{11} & x_{12} & x_{13} \\ x_{21} & x_{22} & x_{23} \\ x_{31} & x_{32} & x_{33} \end{array}$$

(i) For method (1) the following summation notation is used:

$$X_{i.} = \sum_{j} x_{ij} = x_{i1}+x_{i2}+x_{i3},$$

$$X_{.j} = \sum_{i} x_{ij} = x_{1j}+x_{2j}+x_{3j},$$

$$X_{..} = \sum_{i}\sum_{j} x_{ij} = x_{11}+x_{12}+\ .\ .\ .\ +x_{33} \text{ (all nine observations).}$$

(ii) For method (2) the following summation notation is used:

$$X_{i.} = \sum_{j} x_{ij} = x_{i1}+x_{i2}+x_{i3},$$

where $x_{ij} = x_{ji}$; $X_{.j}$ need not be considered.

$$X_{..} = \sum_{i \leqslant j}\sum x_{ij} = x_{11}+x_{12}+x_{13}+x_{22}+x_{23}+x_{33}.$$

(iii) For method (3) the following summation notation is used:

$$X_{i.} = \sum_{j \neq i} x_{ij}, \text{ e.g. } X_{2.} = x_{21}+x_{23}.$$

$$X_{.j} = \sum_{i \neq j} x_{ij}, \text{ e.g. } X_{.2} = x_{12}+x_{32}.$$

$$X_{..} = \sum_{i \neq j}\sum x_{ij} = x_{12}+x_{13}+x_{21}+x_{23}+x_{31}+x_{32}.$$

(iv) For method (4) the following summation notation is used:

$$X_{i.} = \sum_{j \neq i} x_{ij},$$

where $x_{ij} = x_{ji}$, e.g. $X_{2.} = x_{12}+x_{23}$.

$X_{.j}$ need not be considered.

$$X_{..} = \sum_{i<j}\sum x_{ij} = x_{12}+x_{13}+x_{23}.$$

(b) *Statistical Analyses*

Let us assume that one of the four diallel crossing systems has been employed to obtain the experimental material, and that this experimental material has been grown in a randomized-block design in which there are a varieties (genotypes), b blocks, and c observations for each of the ab plots.

The test for differences among genotypes is made by either of the following F tests. For model I, use

$$F_{[(a-1),\, m]} = M_v/M_e,$$

where $(a-1)$ and m are the degrees of freedom associated with the numerator and denominator of the F ratio, and M_v and M_e are the variety and error mean squares respectively in the randomized-block analysis.

For model II, use

$$F_{[(a-1),\, (a-1)(b-1)]} = M_v/M_{bv},$$

where M_{bv} is the variety × block interaction mean square.

If significant F ratios occur we reject the null hypothesis and assume that there are genotypic differences, which may be investigated further with the appropriate combining ability analysis.

(i) *Experimental Method* 1 (*parents, one set of F_1's and reciprocal F_1's are included*).—This diallel crossing system gives rise to p^2 genotypes. Therefore in the randomized-block analysis $a = p^2$.

The combining ability analysis of variance is given in Table 2 (see also Hayman (1954)). To give the estimation and testing procedures we must consider the analyses based on models I and II separately.

Model I

The mathematical model for the combining ability analysis is assumed to be

$$x_{ij} = u+g_i+g_j+s_{ij}+r_{ij}+\frac{1}{bc}\sum_k\sum_l e_{ijkl} \qquad \begin{cases} i, j = 1, \ldots, p, \\ k = 1, \ldots, b, \\ l = 1, \ldots, c, \end{cases}$$

where u is the population mean, $g_i(g_j)$ is the g.c.a. effect for the ith (jth) parents, s_{ij} is the s.c.a. effect for the cross between the ith and jth parents such that $s_{ij} = s_{ji}$, r_{ij} is the reciprocal effect involving the reciprocal crosses between the ith and jth parents such that $r_{ij} = -r_{ji}$, and e_{ijkl} is the environmental effect associated with the $ijkl$th individual observation. The following restrictions are imposed on the combining ability elements: $\sum_i g_i = 0$ and $\sum_i s_{ij} = 0$ (for each j).

The expectations of mean squares are given in Table 2 in the column designated model I. It should be noticed that in this analysis

$$M_e' = M_e/bc,$$

where M_e is the error mean square for the randomized-block design and the expectation of M_e' is denoted as

$$E(M_e') = \sigma_e^2/bc = \sigma^2.$$

TABLE 2

ANALYSIS OF VARIANCE FOR METHOD 1 GIVING EXPECTATIONS OF MEAN SQUARES FOR THE ASSUMPTIONS OF MODELS I AND II

Source	D.F.	Sum of Squares*	Mean Squares	Expectation of Mean Squares	
				Model I	Model II
General combing ability	$p-1$	S_g	M_g	$\sigma^2+2p\left(\frac{1}{p-1}\right)\Sigma g_i^2$	$\sigma^2+\frac{2(p-1)}{p}\sigma_s^2+2p\sigma_g^2$
Specific combining ability	$p(p-1)/2$	S_s	M_s	$\sigma^2+\frac{2}{p(p-1)}\underset{i}{\Sigma}\underset{j}{\Sigma}s_{ij}^2$	$\sigma^2+\frac{2(p^2-p+1)}{p^2}\sigma_s^2$
Reciprocal effects	$p(p-1)/2$	S_r	M_r	$\sigma^2+2\left(\frac{2}{p(p-1)}\right)\underset{i<j}{\Sigma\ \Sigma}r_{ij}^2$	$\sigma^2+2\sigma_r^2$
Error	m	S_e	M_e'	σ^2	σ^2

* Where

$$S_g = \frac{1}{2p}\underset{i}{\Sigma}(X_{i.}+X_{.i})^2-\frac{2}{p^2}X_{..}^2,$$

$$S_s = \frac{1}{2}\underset{i}{\Sigma}\underset{j}{\Sigma}x_{ij}(x_{ij}+x_{ji})-\frac{1}{2p}\underset{i}{\Sigma}(X_{.i}+X_{i.})^2+\frac{1}{p^2}X_{..}^2,$$

$$S_k = \frac{1}{2}\underset{i<j}{\Sigma\ \Sigma}(x_{ij}-x_{ji})^2.$$

Testing for overall differences among the various classes of effects can be accomplished as follows:

(1) To test g.c.a. effects use

$$F_{[(p-1),\, m]} = M_g/M_e'.$$

(2) To test s.c.a. effects use

$$F_{[p(p-1)/2,\, m]} = M_s/M_e'.$$

(3) To test for reciprocal effects use

$$F_{[p(p-1)/2,\, m]} = M_r/M_e'.$$

The various effects may be estimated as follows:

$$\hat{u} = \frac{1}{p^2}X_{..},$$

$$\hat{g}_i = \frac{1}{2p}(X_{i.}+X_{.i})-\frac{1}{p^2}X_{..},$$

$$\hat{s}_{ij} = \frac{1}{2}(x_{ij}+x_{ji})-\frac{1}{2p}(X_{i.}+X_{.i}+X_{j.}+X_{.j})+\frac{1}{p^2}X_{..},$$

and,

$$\hat{r}_{ij} = \frac{1}{2}(x_{ij} - x_{ji}).$$

The variance of any parent or F_1 mean value is

$$\text{var}(x_{ij}) = \hat{\sigma}^2 = M_e',$$

and the variance of the difference between any two mean values is

$$\text{var}(x_{ij} - x_{kl}) = 2\hat{\sigma}^2.$$

Variances of effects and of differences between effects may be estimated as follows:

$$\text{var}(\hat{u}) = \frac{1}{p^2}\hat{\sigma}^2,$$

$$\text{var}(\hat{g}_i) = \frac{p-1}{2p^2}\hat{\sigma}^2,$$

$$\text{var}(\hat{s}_{ii}) = \frac{(p-1)^2}{p^2}\hat{\sigma}^2,$$

$$\text{var}(\hat{s}_{ij}) = \frac{1}{2p^2}(p^2 - 2p + 2)\hat{\sigma}^2 \qquad (i \neq j),$$

$$\text{var}(\hat{r}_{ij}) = \frac{1}{2}\hat{\sigma}^2 \qquad (i \neq j),$$

$$\text{var}(\hat{g}_i - \hat{g}_j) = \frac{1}{p}\hat{\sigma}^2 \qquad (i \neq j),$$

$$\text{var}(\hat{s}_{ii} - \hat{s}_{jj}) = \frac{2(p-2)}{p}\hat{\sigma}^2 \qquad (i \neq j),$$

$$\text{var}(\hat{s}_{ii} - \hat{s}_{ij}) = \frac{1}{2p}(3p-2)\hat{\sigma}^2 \qquad (i \neq j),$$

$$\text{var}(\hat{s}_{ii} - \hat{s}_{jk}) = \frac{3}{2p}(p-2)\hat{\sigma}^2 \qquad (i \neq j,\ k;\ j \neq k),$$

$$\text{var}(\hat{s}_{ij} - \hat{s}_{ik}) = \frac{p-1}{p}\hat{\sigma}^2 \qquad (i \neq j,\ k;\ j \neq k),$$

$$\text{var}(\hat{s}_{ij} - \hat{s}_{kl}) = \frac{p-2}{p}\hat{\sigma}^2 \qquad (i \neq j,\ k,\ l;\ j \neq k,\ l;\ k \neq l),$$

$$\text{var}(\hat{r}_{ij} - \hat{r}_{kl}) = \hat{\sigma}^2 \qquad (i \neq j;\ k \neq l).$$

Model II

The mathematical model for the combining ability analysis is

$$x_{ij} = u + g_i + g_j + s_{ij} + r_{ij} + \frac{1}{b}\sum_k b_k + \frac{1}{b}\sum_k (bv)_{ijk} + \frac{1}{bc}\sum_k \sum_l e_{ijkl},$$

where all effects except u are considered to be random variables.

The expectations of combining ability mean squares are given in Table 2 in the column designated model II. In this analysis the expectation of M_e' is

$$E(M_e') = \frac{1}{bc}(\sigma_e^2 + c\sigma_{bv}^2) = \sigma^2.$$

Thus σ^2 is estimated by M_{bv}/bc, where M_{bv} is the block × variety interaction mean square in the randomized-block analysis.

For testing hypotheses pertaining to the variance components, F ratios may again be employed:

(1) To test $\sigma_g^2 = 0$ it is necessary to synthesize a new mean square, M^* say, defined by

$$M^* = (1-A)M_e' + AM_s,$$

where

$$A = \frac{p(p-1)}{p^2-p+1}.$$

Then the approximate F test

$$F_{[(p-1),\,f]} = M_g/M^*,$$

may be used. f denotes the degrees of freedom associated with M^*, and is obtained from

$$f = \frac{mp^3(p-1)[p+2(p-1)K]^2}{p^5(p-1)(1-A)^2+2mA^2[p^2+2cK]^2},$$

where

$$K = \frac{p^2}{2c}\left(\frac{M_s-M_e'}{M_e'}\right),$$

and

$$c = p^2-p+1.$$

(2) To test $\sigma_s^2 = 0$ use

$$\mathrm{F}_{[p(p-1)/2,\,m]} = M_s/M_e'.$$

(3) To test $\sigma_r^2 = 0$ use

$$\mathrm{F}_{[p(p-1)/2,\,m]} = M_r/M_e'.$$

Variance components are estimated by equating the observed and expected mean squares in Table 2. The estimates are computed as follows:

$$\hat{\sigma}_g^2 = \frac{1}{2p}\left[M_g - \frac{M_e'+p(p-1)M_s}{c}\right],$$

$$\hat{\sigma}_s^2 = \frac{p^2}{2c}[M_s - M_e'],$$

and

$$\hat{\sigma}_r^2 = \tfrac{1}{2}[M_r - M_e'],$$

where

$$c = p^2-p+1.$$

Approximate estimates of the variances for the variance components may be obtained from the following formulae:

$$\operatorname{var}(\hat{\sigma}_g^2) \cong \frac{1}{2p^2(p-1)}M_g^2+\frac{p-1}{pc^2}M_s^2+\frac{1}{2p^2c^2m}(M_e')^2,$$

$$\operatorname{var}(\hat{\sigma}_s^2) \cong \frac{p^3}{(p-1)c^2}M_s^2+\frac{p^4}{2c^2m}(M_e')^2,$$

$$\operatorname{var}(\hat{\sigma}_r^2) \cong \frac{1}{p(p-1)}M_r^2+\frac{1}{2m}(M_e')^2,$$

and

$$\operatorname{var}(\hat{\sigma}^2) \cong \frac{2}{m}(M_e')^2,$$

where $c = p^2-p+1.$

(ii) *Experimental Method 2 (parents and one set of F_1's are included but not reciprocal F_1's).*—This diallel crossing system gives rise to $p(p+1)/2$ different genotypes. Therefore, in the randomized-block analysis $a = p(p+1)/2$. Table 3 presents an orthogonal partitioning of the variety sum of squares.

TABLE 3

ANALYSIS OF VARIANCE FOR METHOD 2 GIVING EXPECTATIONS OF MEAN SQUARES FOR THE ASSUMPTIONS OF MODELS I AND II

Source	D.F.	Sum of Squares*	Mean Squares	Expectation of Mean Squares	
				Model I	Model II
General combining ability	$p-1$	S_g	M_g	$\sigma^2+(p+2)\left(\frac{1}{p-1}\right)\Sigma g_i^2$	$\sigma^2+\sigma_s^2+(p+2)\sigma_g^2$
Specific combining ability	$p(p-1)/2$	S_s	M_s	$\sigma^2+\frac{2}{p(p-1)}\sum_i \sum_j s_{ij}^2$	$\sigma^2+\sigma_s^2$
Error	m	S_e	M_e'	σ^2	σ^2

* Where

$$S_g = \frac{1}{p+2}\left\{\sum_i (X_{i.}+x_{ii})^2-\frac{4}{p}X_{..}^2\right\},$$

$$S_s = \sum_{i\leqslant j}\sum x_{ij}^2-\frac{1}{p+2}\sum_i (X_{i.}+x_{ii})^2+\frac{2}{(p+1)(p+2)}X_{..}^2.$$

Model I

The mathematical model for the combining ability analysis is assumed to be

$$x_{ij} = u+g_i+g_j+s_{ij}+\frac{1}{bc}\sum_k\sum_l e_{ijkl} \qquad \begin{cases} i, j = 1, \ldots, p, \\ k = 1, \ldots, b, \\ l = 1, \ldots, c, \end{cases}$$

where u is the population mean, $g_i(g_j)$ is the g.c.a. effect, s_{ij} is the s.c.a. effect such that $s_{ij} = s_{ji}$, and e_{ijkl} is the effect peculiar to the $ijkl$th observation. The restrictions

$$\sum_i g_i = 0,$$

and

$$\sum_j s_{ij} + s_{ii} = 0 \text{ (for each } i\text{)},$$

are imposed. As in method 1, model I, $M_e' = M_e/bc$.

The following F ratios may be used to test for g.c.a. and s.c.a. effects. To test for differences among g.c.a. effects use

$$F_{[(p-1),\ m]} = M_g/M_e',$$

and to test for differences among s.c.a. effects use

$$F_{[p(p-1)/2,\ m]} = M_s/M_e'.$$

The effects may be estimated as follows:

$$\hat{u} = \frac{2}{p(p+1)} X_{..},$$

$$\hat{g}_i = \frac{1}{p+2}\left[X_{i.} + x_{ii} - \frac{2}{p} X_{..}\right],$$

and

$$\hat{s}_{ij} = x_{ij} - \frac{1}{p+2}[X_{i.} + x_{ii} + X_{j.} + x_{jj}] + \frac{2}{(p+1)(p+2)} X_{..}.$$

The variance of any parent or F_1 mean value is

$$\operatorname{var}(x_{ij}) = \hat{\sigma}^2 = M_e',$$

and the variance of the difference between any two mean values is

$$\operatorname{var}(x_{ij} - x_{kl}) = 2\hat{\sigma}^2.$$

Variances of effects and of differences between effects may be estimated as follows:

$$\operatorname{var}(\hat{u}) = \frac{2}{p(p+1)} \hat{\sigma}^2,$$

$$\operatorname{var}(\hat{g}_i) = \frac{p-1}{p(p+2)} \hat{\sigma}^2,$$

$$\operatorname{var}(\hat{s}_{ii}) = \frac{p(p-1)}{(p+1)(p+2)} \hat{\sigma}^2,$$

$$\operatorname{var}(\hat{s}_{ij}) = \frac{p^2+p+2}{(p+1)(p+2)} \hat{\sigma}^2 \qquad (i \neq j),$$

$$\operatorname{var}(\hat{g}_i - \hat{g}_j) = \frac{2}{p+2} \hat{\sigma}^2 \qquad (i \neq j),$$

$$\operatorname{var}(\hat{s}_{ii} - \hat{s}_{jj}) = \frac{2(p-2)}{p+2} \hat{\sigma}^2 \qquad (i \neq j),$$

$$\operatorname{var}(\hat{s}_{ij} - \hat{s}_{ik}) = \frac{2(p+1)}{p+2} \hat{\sigma}^2 \qquad (i \neq j, k;\ j \neq k),$$

$$\operatorname{var}(\hat{s}_{ij} - \hat{s}_{kl}) = \frac{2p}{p+2} \hat{\sigma}^2 \qquad (i \neq j, k, l;\ j \neq k, l;\ k \neq l).$$

Model II

The mathematical model for the combining ability analysis is

$$x_{ij} = u+g_i+g_j+s_{ij}+\frac{1}{b}\sum_k b_k+\frac{1}{b}\sum_k (bv)_{ijk}+\frac{1}{bc}\sum_k\sum_l e_{ijkl},$$

where all effects except u are random variables.

The expectations of combining ability mean squares are given in Table 3 in the column designated as model II. In this analysis

$$E(M_e') = \frac{1}{bc}(\sigma_e^2+c\sigma_{bv}^2) = \sigma^2.$$

The following F ratios are used for testing hypotheses pertaining to the different variance components. To test $\sigma_g^2 = 0$ use

$$F_{[(p-1),\, p(p-1)/2]} = M_g/M_s,$$

and to test $\sigma_s^2 = 0$ use

$$F_{[p(p-1)/2,\, m]} = M_s/M_e'.$$

The variance components may be estimated as follows:

$$\hat{\sigma}_g^2 = \frac{1}{p+2}[M_g-M_s],$$

and

$$\hat{\sigma}_s^2 = M_s-M_e'.$$

Approximate estimates of the variances for the variance components may be obtained from the following formulae:

$$\operatorname{var}(\hat{\sigma}_g^2) \cong \frac{2}{(p-1)(p+2)^2}M_g^2+\frac{4}{p(p-1)(p+2)^2}M_s^2,$$

$$\operatorname{var}(\hat{\sigma}_s^2) \cong \frac{4}{p(p-1)}M_s^2+\frac{2}{m}(M_e')^2,$$

and

$$\operatorname{var}(\hat{\sigma}^2) \cong \frac{2}{m}(M_e')^2.$$

(iii) *Experimental Method* 3 (*one set of* F_1*'s and reciprocals are included but not the parents*).—This diallel crossing system gives rise to $a = p(p-1)$ different genotypes which fill all of the cells of a p^2 table except those occurring on the main diagonal.

Table 4 presents the combining ability analysis of variance. The construction of this analysis is given by Yates (1947), and a detailed derivation of the expectations of mean squares, when the effects are considered as random variables, is given by Kempthorne (1952). The appropriate analyses for non-orthogonal data when the effects are random variables is given by Henderson (1948, 1952).

Model I

We assume the model for the combining ability analysis to be

$$x_{ij} = u+g_i+g_j+s_{ij}+r_{ij}+\frac{1}{bc}\sum_k\sum_l e_{ijkl} \qquad \begin{cases} i, j = 1, \dots, p, \\ k = 1, \dots, b, \\ l = 1, \dots, c, \end{cases}$$

where u is the population mean, g_i and g_j are the g.c.a. effects, s_{ij} is the s.c.a. effect such that $s_{ij} = s_{ji}$, r_{ij} is the reciprocal genotypic effect such that $r_{ij} = -r_{ji}$, and e_{ijkl} is the error effect peculiar to the $ijkl$th observation. The restrictions

$$\sum_i g_i = 0$$

and

$$\sum_{i \neq j} s_{ij} = 0 \text{ (for each } j),$$

are imposed on the combining ability effects.

TABLE 4

ANALYSIS OF VARIANCE FOR METHOD 3 GIVING EXPECTATIONS OF MEAN SQUARES FOR THE ASSUMPTIONS OF MODELS I AND II

Source	D.F.	Sum of Squares*	Mean Squares	Expectation of Mean Squares	
				Model I	Model II
General combining ability	$p-1$	S_g	M_g	$\sigma^2+2(p-2)\left(\frac{1}{p-1}\right)\Sigma g_i^2$	$\sigma^2+2\sigma_s^2+2(p-2)\sigma_g^2$
Specific combining ability	$p(p-3)/2$	S_s	M_s	$\sigma^2+2\left(\frac{2}{p(p-3)}\right)\sum_{i<j}\Sigma s_{ij}^2$	$\sigma^2+2\sigma_s^2$
Reciprocal effects	$p(p-1)/2$	S_r	M_r	$\sigma^2+2\left(\frac{2}{p(p-1)}\right)\sum_{i<j}\Sigma r_{ij}^2$	$\sigma^2+2\sigma_r^2$
Error	m	S_e	M_e'	σ^2	σ^2

* Where

$$S_g = \frac{1}{2(p-2)}\Sigma(X_{i.}+X_{.i})^2-\frac{2}{p(p--2)}X_{..}^2,$$

$$S_s = \frac{1}{2}\sum_{i<j}\Sigma(x_{ij}+x_{ji})^2-\frac{1}{2(p-2)}\Sigma(X_{i.}+X_{.i})^2+\frac{1}{(p-1)(p-2)}X_{..}^2,$$

$$S_r = \frac{1}{2}\sum_{i<j}\Sigma(x_{ij}-x_{ji})^2.$$

The expectations of mean squares are given in the column designated model I of Table 4. As in method 1, model I, $M_e' = M_e/bc$.

Differences within classes of effects are tested by F ratios:

(1) To test g.c.a. effects use

$$F_{[(p-1),\, m]} = M_g/M_e'.$$

(2) To test s.c.a. effects use

$$F_{[p(p-3)/2,\, m]} = M_s/M_e'.$$

(3) To test reciprocal effects use

$$F_{[p(p-1)/2,\, m]} = M_r/M_e'.$$

The effects may be estimated as follows:

$$\hat{u} = \frac{1}{p(p-1)}X_{..},$$

$$\hat{g}_i = \frac{1}{2p(p-2)}[p(X_{i.}+X_{.i})-2X_{..}],$$

$$\hat{s}_{ij} = \tfrac{1}{2}(x_{ij}+x_{ji})-\frac{1}{2(p-2)}(X_{i.}+X_{.i}+X_{j.}+X_{.j})+\frac{1}{(p-1)(p-2)}X_{..},$$

and

$$\hat{r}_{ij} = \tfrac{1}{2}(x_{ij}-x_{ji}).$$

The variance of any parent or F_1 mean value is

$$\text{var}(x_{ij}) = \hat{\sigma}^2 = M_e',$$

and the variance of the difference between any two mean values is

$$\text{var}(x_{ij}-x_{kl}) = 2\hat{\sigma}^2.$$

Variances of effects and of differences between effects may be estimated as follows:

$$\text{var}(\hat{u}) = \frac{1}{p(p-1)}\hat{\sigma}^2,$$

$$\text{var}(\hat{g}_i) = \frac{p-1}{2p(p-2)}\hat{\sigma}^2,$$

$$\text{var}(\hat{s}_{ij}) = \frac{p-3}{2(p-1)}\hat{\sigma}^2 \qquad (i\neq j),$$

$$\text{var}(\hat{r}_{ij}) = \tfrac{1}{2}\hat{\sigma}^2 \qquad (i\neq j),$$

$$\text{var}(\hat{g}_i-\hat{g}_j) = \frac{1}{p-2}\hat{\sigma}^2 \qquad (i\neq j),$$

$$\text{var}(\hat{s}_{ij}-\hat{s}_{ik}) = \frac{p-3}{p-2}\hat{\sigma}^2 \qquad (i\neq j,\ k;\ j\neq k),$$

$$\text{var}(\hat{s}_{ij}-\hat{s}_{kl}) = \frac{p-4}{p-2}\hat{\sigma}^2 \qquad (i\neq j,\ k,\ l;\ j\neq k,\ l;\ k\neq l).$$

Model II

The mathematical model for the combining ability analysis is

$$x_{ij} = u+g_i+g_j+s_{ij}+r_{ij}+\frac{1}{b}\sum_k b_k+\frac{1}{b}\sum_k (bv)_{ijk}+\frac{1}{bc}\sum_k\sum_l e_{ijkl},$$

where all effects except u are random variables.

The expectations of mean squares are given in Table 4 in the column designated model II. In this analysis

$$E(M_e') = \frac{1}{bc}(\sigma_e^2+c\sigma_{bv}^2) = \sigma^2.$$

F ratios may be used for testing hypotheses pertaining to the different variance components:

(1) To test $\sigma_g^2 = 0$ use

$$F_{[(p-1),\, p(p-3)/2]} = M_g/M_s.$$

(2) To test $\sigma_s^2 = 0$, use

$$F_{[p(p-3)/2,\, m]} = M_s/M_e'.$$

(3) To test $\sigma_r^2 = 0$ use

$$F_{[p(p-1)/2,\, m]} = M_r/M_e'.$$

The variance components are estimated as follows:

$$\hat{\sigma}_g^2 = \frac{1}{2(p-2)}[M_g - M_s],$$

$$\hat{\sigma}_s^2 = \tfrac{1}{2}[M_s - M_e'],$$

and

$$\hat{\sigma}_r^2 = \tfrac{1}{2}[M_r - M_e'].$$

Approximate estimates of the variances for the variance components may be obtained from the following formulae:

$$\operatorname{var}(\hat{\sigma}_g^2) \cong \frac{1}{2(p-1)(p-2)^2}M_g^2 + \frac{1}{p(p-2)^2(p-3)}M_s^2,$$

$$\operatorname{var}(\hat{\sigma}_s^2) \cong \frac{1}{p(p-3)}M_s^2 + \frac{1}{2m}(M_e')^2,$$

$$\operatorname{var}(\hat{\sigma}_r^2) \cong \frac{1}{p(p-1)}M_r^2 + \frac{1}{2m}(M_e')^2,$$

and

$$\operatorname{var}(\hat{\sigma}^2) \cong \frac{2}{m}(M_e')^2.$$

(iv) *Experimental Method* 4 (*one set of F_1's but neither parents nor reciprocal F_1's is included*).—In this, the most common of the diallel crossing systems, there are $a = p(p-1)/2$ different F_1 mean values.

The combining ability analysis of variance is given in Table 5. The analysis was originally presented by Sprague and Tatum (1942).

Model I

The model for the combining ability analysis is

$$x_{ij} = u + g_i + g_j + s_{ij} + \frac{1}{bc}\sum_k \sum_l e_{ijkl} \qquad \begin{cases} i, j = 1, \ldots, p, \\ k = 1, \ldots, b, \\ l = 1, \ldots, c, \end{cases}$$

where u is the population mean, g_i and g_j are the g.c.a. effects, s_{ij} is the s.c.a. effect such that $s_{ij} = s_{ji}$, and e_{ijkl} is the error effect peculiar to the $ijkl$th observation.

The restrictions

$$\sum_i g_i = 0,$$

and

$$\sum_{i \neq j} s_{ij} = 0 \text{ (for each } j\text{)},$$

are imposed on the combining ability effects.

TABLE 5

ANALYSIS OF VARIANCE FOR METHOD 4 GIVING EXPECTATIONS OF MEAN SQUARES FOR THE ASSUMPTIONS OF MODELS I AND II

Source	D.F.	Sum of Squares*	Mean Squares	Expectation of Mean Squares	
				Model I	Model II
General combining ability	$p-1$	S_g	M_g	$\sigma^2+(p-2)\left(\frac{1}{p-1}\right)\sum_i g_i^2$	$\sigma^2+\sigma_s^2+(p-2)\sigma_g^2$
Specific combining ability	$p(p-3)/2$	S_s	M_s	$\sigma^2+\left(\frac{2}{p(p-3)}\right)\sum_{i<j}\sum s_{ij}^2$	$\sigma^2+\sigma_s^2$
Error	m	S_e	M_e'	σ^2	σ^2

* Where

$$S_g = \frac{1}{p-2}\sum_i X_{i.}^2 - \frac{4}{p(p-2)}X_{..}^2,$$

$$S_s = \sum_{i<j}\sum x_{ij}^2 - \frac{1}{p-2}\sum X_{i.}^2 + \frac{2}{(p-1)(p-2)}X_{..}^2.$$

The expectations of mean squares are given in the column designated model I of Table 5. In this analysis

$$M_e' = M_e/bc.$$

Differences within classes of effects are tested by F ratios.

(1) To test g.c.a. effects use

$$F_{[(p-1),\, m]} = M_g/M_e'.$$

(2) To test s.c.a. effects use

$$F_{[p(p-3)/2,\, m]} = M_s/M_e'.$$

The effects may be estimated as follows:

$$\hat{u} = \frac{2}{p(p-1)}X_{..},$$

$$\hat{g}_i = \frac{1}{p(p-2)}[pX_{i.} - 2X_{..}],$$

and

$$\hat{s}_{ij} = x_{ij} - \frac{1}{p-2}(X_{i.}+X_{j.}) + \frac{2}{(p-1)(p-2)}X_{..}.$$

The variance of any F_1 mean value is

$$\operatorname{var}(x_{ij}) = \hat{\sigma}^2 = M_e',$$

and the variance of a difference between any two mean values is

$$\operatorname{var}(x_{ij}-x_{kl}) = 2\hat{\sigma}^2.$$

Variances of effects and of differences between effects may be estimated as follows:

$$\operatorname{var}(\hat{u}) = \frac{2}{p(p-1)}\hat{\sigma}^2,$$

$$\operatorname{var}(\hat{g}_i) = \frac{p-1}{p(p-2)}\hat{\sigma}^2,$$

$$\operatorname{var}(\hat{s}_{ij}) = \frac{p-3}{p-1}\hat{\sigma}^2 \qquad (i\neq j),$$

$$\operatorname{var}(\hat{g}_i-\hat{g}_j) = \frac{2}{p-2}\hat{\sigma}^2 \qquad (i\neq j),$$

$$\operatorname{var}(\hat{s}_{ij}-\hat{s}_{ik}) = \frac{2(p-3)}{p-2}\hat{\sigma}^2 \qquad (i\neq j,\, k;\; j\neq k),$$

$$\operatorname{var}(\hat{s}_{ij}-\hat{s}_{kl}) = \frac{2(p-4)}{p-2}\hat{\sigma}^2 \qquad (i\neq j,\, k,\, l;\; j\neq k,\, l;\; k\neq l).$$

Model II

The model for the combining ability analysis is

$$x_{ij} = u+g_i+g_j+s_{ij}+\frac{1}{b}\sum_k b_k+\frac{1}{b}\sum_k (bv)_{ijk}+\frac{1}{bc}\sum_k\sum_l e_{ijkl},$$

where all effects except u are random variables.

The expectations of mean squares are given in Table 5 in the column designated model II. As with other model II analyses

$$E(M_e') = \frac{1}{bc}(\sigma_e^2+c\sigma_{bv}^2) = \sigma^2.$$

F ratios may be used to test hypotheses pertaining to the variance components:

(1) To test $\sigma_g^2 = 0$ use

$$F_{[(p-1),\, p(p-3)/2]} = M_g/M_s.$$

(2) To test $\sigma_s^2 = 0$ use

$$F_{[p(p-3)/2,\, m]} = M_s/M_e'.$$

The variance components are estimated as follows:

$$\hat{\sigma}_g^2 = \frac{1}{p-2}[M_g-M_s],$$

and

$$\hat{\sigma}_s^2 = M_s-M_e'.$$

Approximate estimates of the variances for the variance components may be obtained as follows:

$$\operatorname{var}(\hat{\sigma}_g^2) \cong \frac{2}{(p-1)(p-2)^2}M_g^2+\frac{4}{p(p-2)^2(p-3)}M_s^2,$$

$$\operatorname{var}(\hat{\sigma}_s^2) \cong \frac{4}{p(p-3)}M_s^2+\frac{2}{m}(M_e')^2,$$

and

$$\operatorname{var}(\hat{\sigma}^2) \cong \frac{2}{m}(M_e')^2.$$

(c) *Use of Mixed Models*

Mixed model A can be used for all four diallel crossing systems. In those diallel crossing systems in which reciprocal F_1's are not included the mathematical model for the combining ability analysis is

$$x_{ij} = u+g_i+g_j+s_{ij}+\frac{1}{bc}\sum_k\sum_l e_{ijkl},$$

and for those diallel systems in which the reciprocal F_1's are included the model is

$$x_{ij} = u+g_i+g_j+s_{ij}+r_{ij}+\frac{1}{bc}\sum_k\sum_l e_{ijkl}.$$

In both models all elements except u are random variables.

The combining ability analyses are the same as those involving model II except that $M_e' = \hat{\sigma}^2 = M_e/bc$ is used as the error mean square.

Use of mixed model B introduces the "mixed" elements $(bv)_{ijk}$ into the combining ability analyses. Thus, for the diallel crossing systems which do not include reciprocal F_1's the model is

$$x_{ij} = u+g_i+g_j+s_{ij}+\frac{1}{b}\sum_k b_k+\frac{1}{b}\sum_k (bv)_{ijk}+\frac{1}{bc}\sum_k\sum_l e_{ijkl}.$$

When the reciprocal F_1's are included the model is

$$x_{ij} = u+g_i+g_j+s_{ij}+r_{ij}+\frac{1}{b}\sum_k b_k+\frac{1}{b}\sum_k (bv)_{ijk}+\frac{1}{bc}\sum_k\sum_l e_{ijkl}.$$

In both models the restrictions

$$\sum_i g_i = 0,$$

$$\sum_i s_{ij} = 0 \text{ (for each } j),$$

and

$$\sum_{i<j}\sum (bv)_{ijk} = 0$$

are imposed.

The introduction of the mixed elements into the combining ability analyses causes difficulty. When mixed model B is used, somewhat arbitrary and inconsistent assumptions must be made with regard to the mixed elements in order to obtain manageable expectations of mean squares and variances. However, if mixed model B

is used, the combining ability analyses are essentially the same as model I analyses, except that $M_e' = M_{bv}/bc$.

(*d*) *Extension to more than One Variable*

No difficulty arises in the simultaneous analysis of two or more variables. In fact in those combining ability analyses employing model II it is possible to use variance and covariance components to estimate the population regression and correlation coefficients for any type of effect in the model. Thus the regression of the ith variable on the jth variable for the θ effect is estimated as

$$b_{\theta_{ij}} = \hat{\sigma}_{\theta_{ij}}{}^2/\hat{\sigma}_{\theta_j}{}^2.$$

The correlation coefficient between the ith and jth variables for the θ effect is estimated as

$$r_{\theta_{ij}} = \hat{\sigma}_{\theta_{ij}}{}^2/\sqrt{\{(\hat{\sigma}_{\theta_i}{}^2)(\hat{\sigma}_{\theta_j}{}^2)\}},$$

where $\hat{\sigma}_{\theta_{ij}}{}^2$ = covariance component for the θ effect involving the ith and jth variables,

$\hat{\sigma}_{\theta_i}{}^2$ = variance component for the θ effect for the ith variable,

and

θ = phenotypic, genotypic, g.c.a., s.c.a., reciprocal, or error effects.

This procedure is particularly useful in plant and animal breeding and is a direct consequence of the earlier notions of the partitioning of the genotypic variance and of discriminant functions, both developed by Fisher (1918, 1936). (The direct application of discriminant functions to plant selection was first made by Smith (1936).) Hazel, Baker, and Reinmiller (1943) made an early application of the use of variance components to estimate correlation coefficients in an animal breeding problem, and an application of the technique in an analysis of tomato yield was made by the author (1948, 1953).

The distribution of such statistics computed from variance and covariance components is not known, and therefore their standard errors cannot be calculated.

III. NUMERICAL EXAMPLE

Space does not permit a detailed example to be given for each of the eight possible analyses. Therefore, attention shall be concentrated on experimental method 4 and a numerical example will be given to illustrate the two analyses arising from models I and II.

Method 4 is chosen for the following reasons:

(1) It represents the most commonly used diallel crossing system.

(2) When the necessary assumptions concerning the sampling nature of the set of inbreds can be validly made, it is possible to give an exact genetic interpretation to the general and specific combining ability variances.

(3) Reciprocal genotypic effects are generally non-existent in plant data of the sort we wish to consider in the illustrative example and therefore it is not necessary to introduce the additional complication of reciprocal F_1's.

For convenience we shall use the same set of data to illustrate the two analyses associated with models I and II. This is done to focus attention more clearly on the differences in analysis and interpretation which result from the two sets of assumptions. In reality the set of lines used in the example were deliberately chosen and, therefore, the analysis based on model I is appropriate. However, for illustrative purposes we shall also present the analysis based on model II.

Table 6

F_1 MEAN VALUES FOR X_1 = TOTAL YIELD, X_2 = COB WEIGHT, AND X_3 = SHELLED CORN WEIGHT

Parent Number	Variable	Parent Number							
		2	3	4	5	6	7	8	9
1	X_1	240·0	260·0	230·4	257·0	241·5	266·9	240·1	300·4
	X_2	31·8	34·7	32·3	45·0	39·0	35·1	35·7	40·1
	X_3	208·2	225·3	198·1	212·0	202·5	231·8	204·4	260·3
2	X_1		209·0	217·3	233·1	229·5	266·9	216·3	214·2
	X_2		27·9	30·8	39·6	33·1	30·9	30·9	28·5
	X_3		181·1	186·5	193·5	196·4	236·0	185·4	185·7
3	X_1			183·7	253·7	250·1	268·8	222·3	252·1
	X_2			25·2	41·4	35·5	34·9	32·1	32·4
	X_3			158·5	212·3	214·6	233·9	190·2	219·7
4	X_1				233·8	213·7	255·7	197·4	281·0
	X_2				42·6	35·7	35·6	32·7	41·3
	X_3				191·2	178·0	220·1	164·7	239·7
5	X_1					206·8	272·2	242·9	260·8
	X_2					40·1	43·6	41·8	44·2
	X_3					166·7	228·6	201·1	216·6
6	X_1						261·8	270·3	283·9
	X_2						39·1	43·5	41·5
	X_3						222·7	226·8	242·4
7	X_1							273·2	302·2
	X_2							38·3	41·1
	X_3							234·9	261·1
8	X_1								259·8
	X_2								35·2
	X_3								224·6

For the example, maize yield data previously reported by Griffing and Lindstrom (1954) will be used. From this study we shall select data from the 36 F_1's resulting from diallel crossing among nine inbred lines. The variables to be considered are X_1 = total yield, X_2 = cob weight, and X_3 = shelled corn weight.

These variables are related: $X_1 = X_2+X_3$. The mean F_1 values for these three variables are given in Table 6.

The experimental design consisted of six randomized blocks. In each block each F_1 plot contained 13 plants. After thinning, the plants were spaced singly and approximately 1 ft apart. The ears were bagged soon after silking to minimize loss.

The first stage in the analysis is to test the null hypotheses that there are no genotypic differences among the F_1 genotypes. These tests involve the randomized-block analyses of variance and covariance. For these analyses we note that $a = 36$, $b = 6$, and $c = 13$. The observed mean squares for X_1, X_2, and X_3 and mean cross-products for X_2, X_3 are given in Table 7. For model I we use $F_{[35,\,2558]} = M_v/M_e$, and for model II we use $F_{[35,\,175]} = M_v/M_{bv}$. Since highly significant F ratios are obtained for all variables we reject the null hypotheses and assume that genotypic differences exist. We are now prepared to continue with the combining ability analyses.

TABLE 7

OBSERVED MEAN SQUARES FROM RANDOMIZED-BLOCKS ANALYSES OF VARIANCE FOR VARIABLES X_1 = TOTAL YIELD, X_2 = COB WEIGHT, AND X_3 = SHELLED CORN WEIGHT, AND MEAN CROSS-PRODUCTS FOR VARIABLES X_2 AND X_3

Source	D.F.	Mean Squares			Mean Cross-products X_2X_3
		X_1	X_2	X_3	
Varieties	35	61,889·36	2,039·31	50,422·97	4,713·54
Blocks	5	257,943·53	2,596·49	210,901·86	22,222·59
Varieties × blocks	175	11,803·61	235·82	9,056·45	1,255·67
Error	2558	1,642·48	46·61	1,247·39	174·24

Model I

The mathematical model for the combining ability analysis is

$$x_{ij} = u+g_i+g_j+s_{ij}+\frac{1}{78}\sum_k\sum_l e_{ijkl} \qquad \begin{cases} i, j = 1, \ldots, 9, \\ k = 1, \ldots, 6, \\ l = 1, \ldots, 13, \end{cases}$$

where the definitions of the elements and the restrictions imposed on them are given in method 4, model I.

The combining ability analyses of variance and covariance are given in Table 8. Highly significant F ratios occur in all variables for both general and specific combining ability effects. Therefore, we assume that true differences among these effects do occur. It may be noted that in these analyses the mean squares and mean cross-products (involving X_2, X_3) are related as follows:

$$_1M_i = {_2M_i}+{_3M_i}+2({_{23}CP_i}),$$

where $_1M_i$ is the mean square for the ith source of variation involving variable X_1, etc., and $_{23}CP_i$ is the mean cross-product for the ith source of variation involving variables X_2 and X_3.

Table 8

MEAN SQUARES AND CROSS-PRODUCTS FROM COMBINING ABILITY ANALYSES, BASED ON MODEL I, FOR VARIABLES X_1 = TOTAL YIELD, X_2 = COB WEIGHT, AND X_3 = SHELLED CORN WEIGHT

Source	D.F.	Mean Squares			Mean Cross-products X_2X_3
		X_1	X_2	X_3	
General combining ability	8	2325·74**	90·98**	1948·66**	143·05**
Specific combining ability	27	339·44**	6·99**	260·49**	35·98**
Error	2558	21·05	0·60	15·99	2·23

Components	X_1	X_2	X_3	X_2X_3
$\frac{1}{8}\widehat{\Sigma g_i^2}$	329·24	12·91	276·09	20·12
$\frac{1}{27}\widehat{\underset{i<j}{\Sigma\,\Sigma}\, s_{ij}^2}$	318·39	6·39	244·50	33·75
$\hat{\sigma}^2$	21·05	0·60	15·99	2·23

$^{**}P<0{\cdot}01$.

Table 9

ESTIMATES OF GENERAL COMBINING ABILITY EFFECTS ($_1\hat{g}_i$, $_2\hat{g}_i$, AND $_3\hat{g}_i$) FOR VARIABLES X_1 = TOTAL YIELD, X_2 = COB WEIGHT, AND X_3 = SHELLED CORN WEIGHT

Parent	General Combining Ability Effects		
	$_1\hat{g}_i$	$_2\hat{g}_i$	$_3\hat{g}_i$
1 (standard)	9·35	0·27	9·08
2	−20·65	−5·48	−15·17
3	−10·16	−3·96	−6·20
4	−22·55	−2·23	−20·32
5	−1·51	6·64	−8·15
6	−1·89	2·24	−4·13
7	28·12	0·97	27·15
8	−6·93	−0·23	−6·70
9	26·22	1·78	24·44
S.E. $(\hat{g}_i-\hat{g}_j)$	2·45	0·41	2·14

The next step is the estimation of effects. This can be done for each variable using the formulae given in method 4, model I, by letting $p = 9$. The g.c.a. estimates

TABLE 10

ESTIMATES OF SPECIFIC COMBINING ABILITY CONSTANTS ($\hat{s}_{ij}$) FOR VARIABLES X_1 = TOTAL YIELD, X_2 = COB WEIGHT, AND X_3 = SHELLED CORN WEIGHT

Parent Number	Trait	Parent Number							
		2	3	4	5	6	7	8	9
1	X_1	4·94	14·46	−2·76	2·80	−12·31	−16·93	−8·67	18·47
	X_2	0·53	1·91	−2·21	1·61	0·01	−2·61	−0·81	1·57
	X_3	4·41	12·55	−0·55	1·19	−12·32	−14·32	−7·86	16·90
2	X_1		−6·54	14·14	8·90	5·68	13·07	−2·47	−37·73
	X_2		0·86	2·03	1·96	−0·14	−1·07	0·13	−4·29
	X_3		−7·40	12·11	6·94	5·82	14·14	−2·60	−33·44
3	X_1			−29·94	19·01	15·80	4·48	−6·96	−10·31
	X_2			−5·09	2·25	0·74	1·41	−0·19	−1·89
	X_3			−24·85	16·76	15·06	3·07	−6·77	−8·42
4	X_1				11·50	−8·21	3·77	−19·47	30·97
	X_2				1·71	−0·79	0·39	−1·31	5·27
	X_3				9·79	−7·42	3·38	−18·16	25·70
5	X_1					−36·16	−0·77	4·99	−10·27
	X_2					−5·25	−0·49	−1·09	−0·70
	X_3					−30·91	−0·28	6·08	−9·57
6	X_1						−10·78	32·77	13·22
	X_2						−0·59	5·01	1·00
	X_3						−10·19	27·76	12·22
7	X_1							5·66	1·50
	X_2							1·09	1·87
	X_3							4·57	−0·37
8	X_1								−5·85
	X_2								−2·83
	X_3								−3·02

Standard Error	X_1	X_2	X_3	Limitations
S.E.$(\hat{s}_{ij}-\hat{s}_{ik})$	6·01	1·01	5·24	$i \neq j,\ k;\ j \neq k$
S.E.$(\hat{s}_{ij}-\hat{s}_{kl})$	5·48	0·93	4·78	$i \neq j,\ k,\ l;\ j \neq k,\ l;\ k \neq l$

are given in Table 9 and the s.c.a. estimates are given in Table 10. For any one variable each F_1 mean value can be represented by a linear function of these effects, i.e.

$$x_{ij} = \hat{u} + \hat{g}_i + \hat{g}_j + \hat{s}_{ij}.$$

Table 11

ESTIMATES OF GENERAL AND SPECIFIC COMBINING ABILITY VARIANCES ASSOCIATED WITH EACH PARENT, AND ESTIMATES OF THE ENVIRONMENTAL VARIANCES ON INDIVIDUAL AND MEAN BASES

Parent	Variable	$\hat{\sigma}_{g_i}^{2}$ *	$\hat{\sigma}_{s_i}^{2}$ *	Individual Basis ($\hat{\sigma}_e^2$)	Mean Basis ($\hat{\sigma}^2$)
1	X_1	84·75	139·60	1642·48	21·05
	X_2	0	2·54	46·61	0·60
	X_3	80·41	112·42	1247·39	15·99
	X_2X_3	2·17	12·32	174·24	2·23
2	X_1	423·75	264·69	1642·48	21·05
	X_2	29·95	3·57	46·61	0·60
	X_3	228·10	218·84	1247·39	15·99
	X_2X_3	82·85	21·14	174·24	2·23
3	X_1	100·55	258·26	1642·48	21·05
	X_2	15·60	5·42	46·61	0·60
	X_3	36·41	195·38	1247·39	15·99
	X_2X_3	24·27	28·73	174·24	2·23
4	X_1	505·83	361·40	1642·48	21·05
	X_2	4·90	9·22	46·61	0·60
	X_3	410·87	260·16	1247·39	15·99
	X_2X_3	45·03	46·01	174·24	2·23
5	X_1	−0·40	270·42	1642·48	21·05
	X_2	44·01	5·76	46·61	0·60
	X_3	64·39	202·06	1247·39	15·99
	X_2X_3	−54·40	31·30	174·24	2·23
6	X_1	0·90	435·28	1642·48	21·05
	X_2	4·94	7·38	46·61	0·60
	X_3	15·02	335·82	1247·39	15·99
	X_2X_3	−9·53	46·04	174·24	2·23
7	X_1	788·06	73·80	1642·48	21·05
	X_2	0·87	1·69	46·61	0·60
	X_3	735·09	64·97	1247·39	15·99
	X_2X_3	26·05	3·57	174·24	2·23
8	X_1	45·36	221·08	1642·48	21·05
	X_2	−0·02	4·91	46·61	0·60
	X_3	42·86	169·39	1247·39	15·99
	X_2X_3	1·26	23·39	174·24	2·23
9	X_1	684·81	431·51	1642·48	21·05
	X_2	3·09	8·81	46·61	0·60
	X_3	595·28	327·06	1247·39	15·99
	X_2X_3	43·22	47·82	174·24	2·23

* Where $\sigma_{g_i}^{2} = (\hat{g}_i)^2 - \frac{8}{63}\hat{\sigma}^2$, $\hat{\sigma}_{s_i}^{2} = \frac{1}{7}\sum_j \hat{s}_{ij}^{2} - \frac{6}{7}\hat{\sigma}^2$.

Since the variables are related ($X_1 = X_2+X_3$) we can break down the effects for X_1 into contributions due to X_2 and X_3, i.e.

$${}_1\hat{g}_i = {}_2\hat{g}_i + {}_3\hat{g}_i,$$

and

$${}_1\hat{s}_{ij} = {}_2\hat{s}_{ij} + {}_3\hat{s}_{ij},$$

where the subscript in front of the effect indicates the variable; for example, ${}_2\hat{s}_{ij}$ is the s.c.a. effect involving variable X_2 for the hybrid of the ith and jth parents. In this way each observed value of X_1 can be represented in terms of estimated effects of X_2 and X_3 as follows:

$${}_1x_{ij} = {}_2\hat{u} + {}_3\hat{u} + {}_2\hat{g}_i + {}_3\hat{g}_i + {}_2\hat{g}_j + {}_3\hat{g}_j + {}_2\hat{s}_{ij} + {}_3\hat{s}_{ij}.$$

Standard errors for effects and for differences between effects may be computed by the formulae given in method 4, model I.

Comparisons of the performances of the individual lines is of considerable interest. Clearly the g.c.a. of the lines may be compared directly from Table 9. The next question is, how can the average s.c.a. of the series of crosses involving one line be compared with the average s.c.a. of the series of crosses involving a different line? This may be done by computing

$$\hat{\sigma}_{s_i}^{\;2} = \frac{1}{p-2}\widehat{\sum_j s_{ij}^{\;2}}$$

for each line. The s.c.a. effects may be used to calculate $\hat{\sigma}_{s_i}^{\;2}$ as follows:

$$\hat{\sigma}_{s_i}^{\;2} = \frac{1}{p-2}\sum_{j\neq i}\hat{s}_{ij}^{\;2} - \frac{p-3}{p-2}\hat{\sigma}^2.$$

Values of $\hat{\sigma}_{s_i}^{\;2}$ are given in Table 11.

In addition to comparing values of $\hat{\sigma}_{s_i}^{\;2}$ for different lines it is also informative to compare the relative magnitudes of g.c.a., s.c.a., and error variances. Appropriate statistics for these comparisons are given in Table 11. The combining ability variance associated with the ith line is denoted as

$$\hat{\sigma}_{g_i}^{\;2} = \widehat{(g_i^{\,2})},$$

and may be computed from the g.c.a. estimate g_i as follows:

$$\hat{\sigma}_{g_i}^{\;2} = (\hat{g}_i)^2 - \frac{p-1}{p(p-2)}\hat{\sigma}^2.$$

A brief summary of results indicate these facts. (1) From Table 9 we note that lines 7 and 9 have significantly greater combining ability than line 1 which is taken as the standard. (2) Differences in g.c.a. for total yield (X_1) are largely due to differences in shelled corn weight (X_3). (3) Although lines 7 and 9 are similar in that they both exhibit high g.c.a., they attain their high average performance by entirely different means. The relatively low s.c.a. variance associated with line 7 (i.e. $\hat{\sigma}_{s_7}^{\;2}$, Table 11) indicates that line 7 uniformly transmits its high yielding ability to all of its F_1's, whereas the high s.c.a. variance associated with line 9 indicates that there are specific combinations of line 9 with certain inbreds which yield considerably more than would be expected and other combinations which yield much less than expected. For this reason line 7 is probably superior to line 9 for inclusion in the production of a synthetic variety, and line 9 is probably superior to line 7 if specific high yielding combinations are desired. Both lines 7 and 9 should be tested more thoroughly in order that maximum use of their superior combining ability can be made. (4) In comparing the relative magnitudes of $\hat{\sigma}_{g_i}{}^2$, $\hat{\sigma}_{s_i}{}^2$, and error variances it may be noted that the relatively enormous values of the error variances on an individual basis are brought into manageable magnitudes by the inclusion of a fairly large number (78) of individual observations for each genotype. Thus for X_1 the error variance on an individual basis, $\hat{\sigma}_e{}^2 = 1642{\cdot}48$, is reduced to $\hat{\sigma}_e{}^2/78 = 21{\cdot}05$ on a mean basis. In this way the precision of the estimates of the genotypic effects and variances is enhanced. Even greater precision can be obtained by increasing the number of observations for each genotypic mean.

For a more detailed examination of the results of this experiment the reader is referred to the original paper from which the data were obtained.

Model II

As stated before, in reality the experimental material cannot be regarded as a random sample from some parent population. However, for illustrative purposes we shall assume that they do, and in fact we shall go even farther and pretend that the experimental material constitutes a random sample from a random mating population. By making this assumption we can estimate the additive and non-additive genetic variances of the parent population.

TABLE 12

MEAN SQUARES AND CROSS-PRODUCTS FROM COMBINING ABILITY ANALYSES, BASED ON MODEL II, FOR VARIABLES X_1 = TOTAL YIELD, X_2 = COB WEIGHT, AND X_3 = SHELLED CORN WEIGHT

Source	D.F.	Mean Squares			Mean Cross-products X_2X_3
		X_1	X_2	X_3	
General combining ability	8	2325·75**	90·98**	1948·66**	143·05**
Specific combining ability	27	339·44**	6·99**	260·49**	35·98**
Error	175	151·33	3·02	116·11	16·10

$^{**}P<0{\cdot}01$.

The mathematical model for the combining ability analysis is

$$x_{ij} = u+g_i+g_j+s_{ij}+\frac{1}{6}\sum_k b_k+\frac{1}{6}\sum_k (bv)_{ijk}+\frac{1}{78}\sum_k\sum_l e_{ijkl} \qquad \left\{\begin{array}{l} i, j = 1, \ldots, 9, \\ k = 1, \ldots, 6, \\ l = 1, \ldots, 13, \end{array}\right.$$

where the definitions of the elements are given in method 4, model I.

Using the formulae in Table 5 we can compute the combining ability analyses of variance for variables X_1, X_2, and X_3 and the analysis of covariance for X_2 and X_3. These are given in Table 12. When applying the appropriate F tests we find that there are highly significant differences among g.c.a. and among s.c.a. effects.

From these analyses we may estimate the variance and covariance components by equating the expected to the observed mean squares. These components, or functions of them, are given in Table 13. It may be noted that: (1) the following relation holds for any effect, $\hat{\sigma}_1^2 = \hat{\sigma}_2^2+\hat{\sigma}_3^2+2\hat{\sigma}_{23}^2$ (where $\hat{\sigma}_1^2$, $\hat{\sigma}_2^2$, and $\hat{\sigma}_3^2$ are the estimated components of variance for X_1, X_2 and X_3, respectively, and $\hat{\sigma}_{23}^2$ is the estimated covariance component for X_2, X_3), and (2) estimates of covariances for X_1, X_2, and X_1, X_3 may be obtained by use of the equations $\hat{\sigma}_{12}^2 = \hat{\sigma}_2^2+\hat{\sigma}_{23}^2$ and $\hat{\sigma}_{13}^2 = \hat{\sigma}_3^2+\hat{\sigma}_{23}^2$.

TABLE 13

ESTIMATED VARIANCE AND COVARIANCE COMPONENTS FOR VARIABLES X_1 = TOTAL YIELD, X_2 = COB WEIGHT, AND X_3 = SHELLED CORN WEIGHT

Variable	Component	Variable		
		X_1	X_2	X_3
X_1	$\hat{\sigma}_P^2$	2398·11 ± 342·22	299·30	2098·81
	$\hat{\sigma}_G^2$	755·63 ± 339·13	78·45	677·18
	$2\hat{\sigma}_g^2$	567·52 ± 333·30	54·60	512·92
	$\hat{\sigma}_s^2$	188·11 ± 93·79	23·85	164·26
	$\hat{\sigma}_e^2$	1642·48 ± 45·93	220·85	1421·63
X_2	$\hat{\sigma}_P^2$		74·58 ± 13·14	224·72
	$\hat{\sigma}_G^2$		27·97 ± 13·07	50·48
	$2\hat{\sigma}_g^2$		24·00 ± 13·01	30·60
	$\hat{\sigma}_s^2$		3·97 ± 1·93	19·88
	$\hat{\sigma}_e^2$		46·61 ± 1·30	174·24
X_3	$\hat{\sigma}_P^2$			1874·09 ± 285·36
	$\hat{\sigma}_G^2$			626·70 ± 283·22
	$2\hat{\sigma}_g^2$			482·32 ± 279·12
	$\hat{\sigma}_s^2$			144·38 ± 71·98
	$\hat{\sigma}_e^2$			1247·39 ± 34·88

The population additive genetic and non-additive genetic variances can be estimated from the combining ability components by $\hat{\sigma}_a^2 = 2\hat{\sigma}_g^2$ and $\hat{\sigma}_{na}^2 = \hat{\sigma}_s^2$. (These estimates are unbiased only in the absence of epistatic effects.) The population genotypic and phenotypic variances may be estimated as $\hat{\sigma}_G^2 = 2\hat{\sigma}_g^2+\hat{\sigma}_s^2$ and $\hat{\sigma}_p^2 = \hat{\sigma}_G^2+\hat{\sigma}_e^2$. By estimating $\hat{\sigma}_p^2$ in this way we have disregarded the replication and replication × variety interaction effects and have included as the environmental variation the error variance associated with the individual plant observations.

The formulae for the calculation of approximate estimates of the variances of the variance components $\hat{\sigma}_e^2$ and $\hat{\sigma}_s^2$ were previously given. The corresponding formulae for $2\hat{\sigma}_g^2$, $\hat{\sigma}_G^2$, and $\hat{\sigma}_P^2$ are as follows:

$$\text{var}(2\hat{\sigma}_g^2) \cong \frac{8}{(p-1)(p-2)^2}M_g^2+\frac{16}{p(p-2)^2(p-3)}M_s^2 = 4[\,\text{var}(\hat{\sigma}_g^2)],$$

$$\text{var}(\hat{\sigma}_G^2) \cong \frac{8}{(p-1)(p-2)^2}M_g^2+\frac{4(p-4)^2}{p(p-2)^2(p-3)}M_s^2+\frac{2}{m}M_e^2,$$

and

$$\text{var}(\hat{\sigma}_P^2) \cong \frac{8}{(p-1)(p-2)^2}M_g^2+\frac{4(p-4)^2}{p(p-2)^2(p-3)}M_s^2.$$

The standard errors attached to the estimated variance components in Table 13 are simply the square root of these approximate variances.

As outlined previously it is possible to examine the joint response of two variables for any effect in the model. The estimated correlation coefficients are given in Table 14. These correlation coefficients estimate the joint responses as they occur in the parent random mating population. The genotypic joint responses depend primarily on pleiotropic effects of genes and not on linkage.

TABLE 14

CORRELATION COEFFICIENTS CALCULATED FROM VARIANCE AND COVARIANCE COMPONENTS FOR VARIABLES X_1 = TOTAL YIELD, X_2 = COB WEIGHT, AND X_3 = SHELLED CORN WEIGHT

Variable	Kind of Correlation	X_2	X_3
X_1	r_P	0·71	0·99
	r_G	0·54	0·98
	r_g	0·47	0·98
	r_s	0·87	1·00
	r_e	0·80	0·99
X_2	r_P		0·60
	r_G		0·38
	r_g		0·28
	r_s		0·83
	r_e		0·72

The following illustrates the type of interpretation which may be made concerning these correlated responses, assuming that the experimental material is in fact a random sample from a random mating population.

The genotypic correlation coefficient for variables X_2, X_3, $r_G = 0{\cdot}38$, indicates a slight positive genetic association. On the average a gene causing increased cob weight also causes increased shelled corn weight. This is logical in that, other factors being constant, a gene causing increased cob surface area would make possible a greater number of kernels. However, the fact that the correlation coefficient is not large indicates that there are genetic factors operating in one variable which act independently of the other variable. For example, there may be genes causing variation in size and density of kernels which do not induce variability in cob weight.

On breaking down the genotypic joint response for variables X_2 and X_3 it is of interest to find that the additive and non-additive correlation coefficients, although both positive, differ considerably in magnitude. The additive effects show little association, $r_g = 0{\cdot}28$, and the non-additive effects show a close association, $r_s = 0{\cdot}83$. The small additive genetic correlation coefficient indicates that in the population selection could act on the two variables almost independently.

The large positive environmental correlation coefficient, $r_e = 0{\cdot}72$, may be interpreted to mean that for a given genotype increased nutrients tend to produce an increased cob size which in turn permits a greater shelled corn weight.

When, as above, interpretations of correlation coefficients computed from variance and covariance components are given, it should be pointed out that (1) the joint responses of pairs of variables are assumed linear, and (2) the reliability of the estimates is not known. Since the distribution of such statistics is not known their variances cannot be calculated.

IV. Discussion

The appropriateness of the various kinds of diallel crossing methods depends on the experimental material and the objectives of the experiment.

When information on general and specific combining ability for a specific set of lines is desired in connection with a plant or animal breeding problem, experimental methods 3 or 4 are most applicable. In plant material, if it can be assumed that there will be no genotypic reciprocal effects (this is usually the case) method 4 is most suitable. If there is any doubt about whether or not reciprocal genotypic effects will occur, then both sets of F_1's should be included and diallel crossing method 3 used. In animal experiments, reciprocal crosses should be a fairly standard procedure. The reciprocal effects can be usefully employed to detect variation due to sex-linked genes and maternal effects. Thus method 3 would be most useful.

In most combining ability analyses in which a chosen set of lines is used, interest centres on the performance of F_1's. Therefore, the parental lines need not be included. However, in certain instances it might be advisable to include the parents. For example, if the combining ability analysis is employed to determine suitable lines to combine into a synthetic variety, and if considerable inbreeding occurs in the species, then it is advisable to include the parents and use methods 1 or 2.

In plant and animal breeding problems when a random set of lines is used, diallel crossing methods 3 or 4 are again most applicable. If it is desired to measure reciprocal effects method 3 must be used.

If it can be assumed that the experimental material constitutes a random sample from a population, then it is possible to use the genotypic components of variance and covariance to compute a discriminant function selection index which will aid in the simultaneous selection of several variables. If the further assumption can be made that the experimental material represents a random sample from a random mating population, the additive genetic variance and covariance components may be substituted for the genotypic components.

When a set of inbred lines is used in a diallel crossing system a genetic interpretation in terms of quantitative inheritance is made possible by the fact that the analysis is really a "gamete" combining ability analysis. Thus the genetic properties of a diploid individual may be regarded as the combination of the genetic properties of the two gametes which united to form the individual. Therefore in the statistical analysis we may regard the genotypic effect of an individual as the summation of effects contributed by each gamete (i.e. set of genes in the gamete) and the interaction of gametes (i.e. the interaction of the genes in one gamete with those in the other).

If the experimental material can be assumed to be a random sample from a random mating population, the genetic interpretation can be pushed back to the gene rather than the gamete level, and an exact genetic interpretation may be given to the combining ability statistics (Griffing 1956). For this to be true we must assume that the set of inbred lines used in the diallel crosses is a random set of lines from a hypothetical population of inbred lines, which can be obtained from the parent random mating population by the imposition of an inbreeding system acting in the absence of forces which change gene frequencies. If this chain of assumptions can be validly made, then we can estimate the additive and non-additive genetic variances of the parent population. It should be pointed out that to obtain unbiased estimates, diallel crossing methods 3 or 4 must be used (i.e. the parents must not be included in the *combining ability analysis*). It is advisable, however, to include the parents in the experimental material grown in the experiment so that comparisons of hybrids with their parents can be made in other types of analyses.

In conclusion we see that diallel crossing systems may be used in at least two broad and related fields, namely, those of plant and animal breeding, and quantitative inheritance. The diallel crossing systems in which the parents are not included are generally the most useful in both fields. However, other systems may be used in special situations.

V. Acknowledgments

The author is grateful to Drs. G. S. Watson and F. H. W. Morley for comments on the manuscript, and to Mr. G. A. McIntyre for his construction of the analysis of variance for diallel method 2.

VI. References

Eisenhart, C. (1947).—The assumptions underlying the analysis of variance. *Biometrics* **3**: 1–21.

Fisher, R. A. (1918).—The correlation between relatives on the supposition of Mendelian inheritance. *Trans. Roy. Soc. Edin.* **52**: 399–433.

Fisher, R. A. (1936).—The use of multiple measurements in taxonomic problems. *Ann. Eugen., Camb.* **7**: 179–88.

Griffing, B. (1948).—Statistical analysis of gene action in components of tomato yield. Unpublished Ph.D. Dissertation. (Iowa State College Library, Ames, Iowa.)

Griffing, B. (1953).—An analysis of tomato yield components in terms of genotypic and environmental effects. Bull. Ia. Agric. Exp. Sta. No. 397.

Griffing, B. (1956).—A generalized treatment of diallel crosses in quantitative inheritance. *Heredity* **10**: 31–50.

Griffing, B., and Lindstrom, E. W. (1954).—A study of the combining abilities of corn inbreds having varying proportions of corn belt and non-corn belt germ plasm. *Agron. J.* **46**: 545–52.

Hayman, B. I. (1954).—The analysis of variance of diallel tables. *Biometrics* **10**: 235–44.

Hazel, L. N., Baker, M. L., and Reinmiller, C. F. (1943).—Genetic and environmental correlations between the growth rates of pigs at different ages. *J. Anim. Sci.* **2**: 118–28.

HENDERSON, C. R. (1948).—Estimation of general, specific, and maternal combining abilities in crosses among inbred lines of swine. Unpublished Ph.D. Dissertation. (Iowa State College Library, Ames, Iowa.)

HENDERSON, C. R. (1952).—Specific and general combining ability. In "Heterosis." (Ed. J. W. Gowen.) (Iowa State College Press: Ames.)

KEMPTHORNE, O. (1952).—"Design and Analysis of Experiments." (John Wiley & Sons, Inc.: New York.)

SMITH, H. F. (1936).—A discriminant function for plant selection. *Ann. Eugen., Camb.* **7**: 240–50.

SPRAGUE, G. F., and TATUM, L. A. (1942).—General versus specific combining ability in single crosses of corn. *J. Amer. Soc. Agron.* **34**: 923–32.

YATES, F. (1947).—The analysis of data from all possible reciprocal crosses between a set of parental lines. *Heredity* **1**: 287–301.

THE INHIBITION OF PHOTOSYNTHESIS BY OXYGEN

I. COMPARATIVE PHYSIOLOGY OF THE EFFECT

By J. S. Turner,* Mary Todd,* and E. G. Brittain*

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Summary

(i) A comparative summary is presented of new and published data on the inhibition of photosynthesis by high oxygen concentration.

(ii) An oxygen inhibition of photosynthesis is demonstrated for 25 species, representing the following major plant groups: Angiospermae—land and aquatic forms, both monocotyledons and dicotyledons, Filicales, Bryophyta, Chlorophyceae, Phaeophyceae, Rhodophyceae.

(iii) The inhibition is rapidly produced and equally rapidly reversible in all the species so investigated.

(iv) In the great majority of experiments at high light intensity and high CO_2 concentration the inhibition is not significant until the oxygen concentration exceeds 20 per cent. The effect is absent or small between 0 and 20 per cent., large and variable between 20 and 100 per cent.

(v) The inhibitory effect of oxygen on photosynthesis cannot be accounted for by a higher rate of dark respiration accompanying photosynthesis at the higher oxygen concentrations.

(vi) In two angiosperms, a reduced rate of photosynthesis resulted on transfer of the tissue from 20 to 5 per cent. oxygen. This could have been due to stomatal control of the process, but requires further investigation.

I. Introduction

Although oxygen is a product of the photosynthetic reaction, there has been comparatively little study of the effect of the partial pressure of oxygen on the rate of the reaction. When the present work was initiated, the major publication on this aspect of photosynthesis was that of Warburg (1920). He demonstrated for *Chlorella* a well-marked depression in the rate of photosynthesis when the external gas phase was changed from 2 to 100 per cent. oxygen. Similar oxygen inhibition in *Chlorella* was described 18 years later by Wassink *et al.* (1938). It was also described in the brown alga *Nereocystis* (Surbeck, Holt, and Lund 1925), in *Scenedesmus* (Gaffron 1939, 1940), and in the wheat leaf (McAlister and Myers 1940). Moyse (1953) reported unusual effects of oxygen on the photosynthesis of a crassulacean, *Bryophyllum.* The first thorough study of the typical oxygen inhibition of photosynthesis was that of Tamiya and Huzisige (1949) who worked with *Chlorella ellipsoidea*, but it is to be noted that their conclusions on the mechanism of the effect have been radically altered as a result of further work on the effect of oxygen on the CO_2-fixing capacity of *Chlorella* cells (Miyachi, Izawa, and Tamiya 1955). In the present paper we summarize the published results and relate them to new data gathered over a period of years, in order to show the very widespread occurrence of the effect in the plant kingdom, its extent, and its reversibility.

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The oxygen effect dealt with here apparently bears no relation to the effect of the complete lack of oxygen on photosynthesis. There is evidence (Gaffron 1940, Hill and Whittingham 1953) that even a short dark period of complete anaerobiosis may suppress photosynthesis, alter the lag phase, or change the assimilatory quotient.

We use the following symbols and conventions:

rP = rate of real photosynthesis,
aP = rate of apparent photosynthesis,
Rd = rate of dark respiration.

The concentrations of CO_2 and oxygen quoted are those at the beginning of an experimental period. Unless otherwise stated, percentage inhibition figures are based on the rate in the lower or lowest oxygen concentration applied, e.g.

$$\text{Percentage inhibition (real)} = \{(rP_{N_2}-rP_{O_2})/rP_{N_2}\} \times 100,$$

and

$$\text{Percentage inhibition (apparent)} = \{(aP_{\text{air}}-aP_{O_2})/aP_{\text{air}}\} \times 100.$$

II. Oxygen Effects in Various Species

(a) *Fresh Water Algae*

(i) Chlorella *Sp.* (*probably* C. pyrenoidosa).—Warburg (1920) recorded only 30-min readings in each gas, and did not demonstrate reversibility of the inhibition. He published the results of five such readings in 100 per cent. oxygen, one in air, five in 2 per cent. oxygen. Light intensity was saturating (10,000–20,000 lux, $aP = 20\ Rd$) and CO_2 concentration was high (the cells suspended in carbonate-bicarbonate buffer (No. 9)). In Table 1 and Figure 1, the rates in 2 per cent. oxygen are all converted to 100, and the corresponding figures for higher oxygen concentrations are given. As the effect here is so variable, the single result for 21 per cent. oxygen does not allow one to draw any conclusions as to the form of the aP *v.* $[O_2]$ curve. Percentage inhibition by pure oxygen ranged from 29 to 42.

Table 1

DATA OF WARBURG FOR CHLORELLA SP.

Temperature 25°C; $[CO_2] = 91\times10^{-6}$ mole/l; light intensity 10,000–20,000 lux

Oxygen Concentration (%)	Comparative Rates of Apparent Photosynthesis
100	57·8, 60·9, 67·9, 69·2, 71·1
21	80·2
2	100 (five values, range 45–65, converted)

It was immaterial whether nitrogen or hydrogen was the diluting gas. Warburg stated that there was no clear effect of oxygen concentration on the rate of photosynthesis in low light intensity (400–800 lux, $aP = Rd$), but quoted only two results in support of this. The oxygen effect could not have been due to an acceleration of the normal respiration, for there was no change in the rate of the dark respiration when the oxygen concentration was raised from 2 to 100 per cent.

(ii) Chlorella vulgaris *var.* viridis.—Wassink *et al.* (1938) confirmed some of Warburg's results. At high CO_2 concentration (91×10^{-6} mole/l) and high, but not saturating, light from a sodium lamp ($1{\cdot}45 \times 10^4$ ergs/cm²/sec) they obtained oxygen inhibition of photosynthesis, the rate in oxygen being between 33 and 47 per cent. lower than that in pure nitrogen. Contrary to Warburg's experience, the rates in nitrogen and air were not significantly different (Fig. 1). They used "extra pure nitrogen freed from the last traces of oxygen by. . . . . . . .double reduction over heated copper" but did not incubate the cells in nitrogen previous to illumination (which may increase the lag phase (Gaffron 1940)); they stated that oxygen was evolved after a few minutes illumination, and that the rate of its production remained constant during the experiments. The lack of an oxygen effect between air and nitrogen can hardly be ascribed, therefore, to the extra complications that might ensue as a result of using pure nitrogen. Dark metabolism was taken into account and was very small under all conditions.

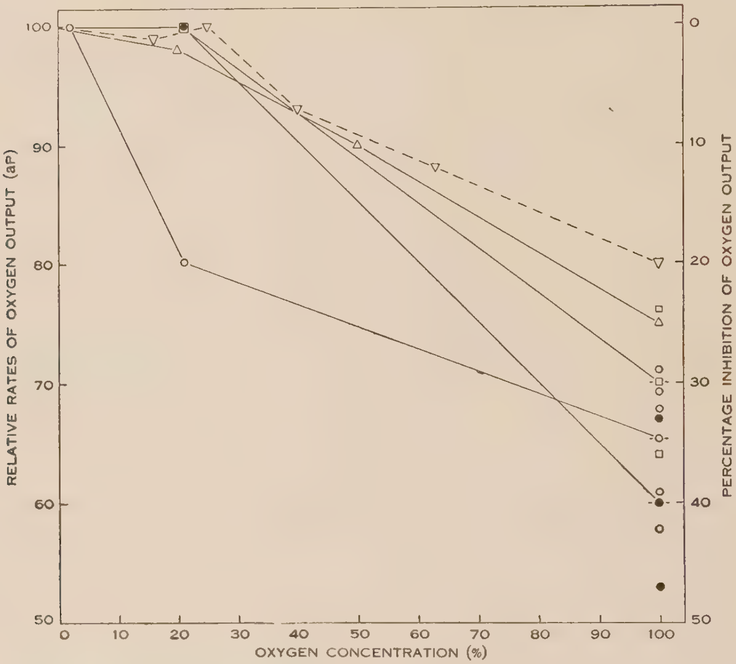

Fig. 1.—Oxygen inhibition of photosynthesis in *Chlorella* as a function of oxygen concentration. ○ (mean —○—) Warburg (1920); ● (mean —●—) Wassink *et al.* (1938); △▽ Tamiya and Huzisige (1944); □ (mean —□—) Briggs and Whittingham (1952).

(iii) Chlorella ellipsoidea.—Tamiya and Huzisige (1949) worked throughout at saturating light intensity (25,000 lux). They referred all inhibitions caused by oxygen to the rate in nitrogen (which was purified by passage over copper at 450°C).

At high light intensity and high CO_2 concentration (91×10^{-6} mole/l) they obtained the results shown in Figure 1. On passage from nitrogen to oxygen, there was between 20 and 25 per cent. decrease in the rate of oxygen output. In agreement with Wassink *et al.*, and differing from Warburg, they obtained only a small oxygen effect (2 per cent., of doubtful significance) between air and nitrogen. They reported a much larger percentage oxygen inhibition at low CO_2 concentration (which will be discussed elsewhere) and it is to be emphasized that under these conditions the inhibition due to the change from air to oxygen was five or six times that due to the change from nitrogen to air.

Tamiya and Huzisige (1949) were the first to publish results showing the reversibility of the oxygen inhibition. Initial rates in air were not recorded, but quantitatively similar effects (but of different sign) were obtained on passage from oxygen to nitrogen (three determinations) or from nitrogen to oxygen (three determinations). Moreover, the rate in nitrogen was the same whether passage was from pure oxygen, 50 per cent. oxygen, or from air. No results were given showing a double change (e.g. oxygen to nitrogen to oxygen) and there were no rate/time curves given for any gas mixture. In a personal communication, however, Professor Tamiya writes that their manometer readings were made at 10–20-min intervals for periods of 60–80 min, during which time the rates were steady.

(iv) Chlorella pyrenoidosa (*Emerson strain*).—Briggs and Whittingham (1952) reported that in this species at high light intensity (40,000 lux) and high CO_2 concentration ($= 91 \times 10^{-6}$ mole/l) there was no oxygen inhibition of aP over the range 0·5–21 per cent. oxygen; there was marked inhibition (30 per cent.) by oxygen over the range 21–100 per cent. oxygen (Fig. 1). The slightly different results for cells "adapted" by a period of illumination in low concentrations of CO_2 will be discussed elsewhere.

(v) Chlorella vulgaris *and* C. pyrenoidosa.—In our experiments we have used the Warburg manometric method but with a different experimental technique from that used in work already reported. Some comparisons have been made between separate, comparable samples shaken in different gas mixtures; in each experiment, however, each sample also served as its own control, being shaken and illuminated successively under two or sometimes three gas mixtures (e.g. 2·5–20–2·5 per cent. oxygen). The light source was a tubular sodium discharge lamp (Osram SD/H82). Typical experiments are illustrated in Figure 2. In such long experiments (2–3 hr), the rate of photosynthesis (aP) rarely remains constant and allowance must be made for drifts in calculating inhibition by oxygen. The details of calculation are indicated in the legend to Figure 2.

All gas exchanges were made with the vessels out of the bath, and gas mixtures were previously equilibrated over the appropriate carbonate-bicarbonate buffer in order to minimize pressure changes due to equilibration in the closed vessel. Only those results obtained at high CO_2 concentration and high light intensity are reported here. In most experiments only apparent photosynthesis rates were measured; the effects of oxygen on dark respiration were small when the gas change was from 20 to 100 per cent. (where the oxygen effect on photosynthesis is greatest), and in these experiments $Rd < aP/10$.

C. vulgaris: Temperature 25°C, $[CO_2] = 37{\cdot}5 \times 10^{-6}$ mole/l, light intensity 30,000 lux (sodium lamp) (Fig. 2(*a*)). A change from air to 100 per cent. oxygen or from 100 per cent. oxygen to air gave 27 and 24 per cent. inhibition of aP by the higher oxygen concentration, the inhibition being reversible.

C. pyrenoidosa: Temperature 25°C, $[CO_2] = 91 \times 10^{-6}$ mole/l, light intensity 30,000 lux (sodium lamp). Three series of experiments were carried out:

(1) Nitrogen to 100 per cent. oxygen (12 expts.): the oxygen inhibition of *aP* was very variable; in two experiments it was as low as 9 and 11 per cent. but in the others it ranged from 22 to 100 per cent., with an overall mean of 35 per cent.

(2) 20 to 100 per cent. oxygen (7 expts.): the oxygen inhibition ranged from 20 to 42 per cent. the mean (30 per cent.) not being significantly different from the mean in (1).

(3) 2·5 to 20 per cent. oxygen (5 expts.): the oxygen inhibition ranged from 0–9 per cent., and was of doubtful significance.

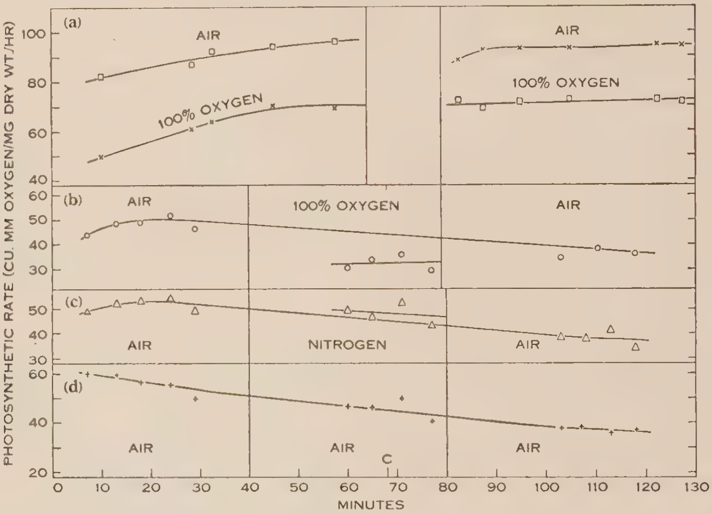

Fig. 2.—Representative experimental results with *Chlorella*. (*a*) *C. vulgaris:* showing reversible inhibition of photosynthesis by oxygen. Percentage effects for comparable samples calculated from the curves at 60 and 100 minutes. Temperature 25°C; $[CO_2] = 37{\cdot}5 \times 10^{-6}$ mole/l, light intensity 30,000 lux. (*b*), (*c*), (*d*) *C. pyrenoidosa:* The drift of apparent photosynthetic rate in air, showing the effect of interpolating short periods of oxygen, nitrogen, or air (renewed). Gas changes made at 40 and 80 minutes. Percentage inhibition calculated from the curves at time *C*. Temperature 25°C; $[CO_2] = 91 \times 10^{-6}$ mole/l; light intensity 30,000 lux.

The oxygen inhibition of *aP* was clearly reversible, but owing to rate/time drifts it is not possible in these experiments to be certain that reversibility was always complete—as it appears to be in Figure 2(*a*).

The above 24 experiments included nine in which the *Chlorella* was grown in low oxygen concentration (0·5 per cent.), five in high oxygen concentration (95 per cent.), and 10 in 21 per cent. oxygen, all gases being enriched with 5 per cent. CO_2. All samples showed clear oxygen inhibition of *aP* on transfer from low to high oxygen concentration. The possibility that cultural conditions and especially oxygen concentration modify the extent of the oxygen inhibition and the growth rate will be discussed in a later paper.

(vi) Scenedesmus "D_3".—Gaffron (1940), in the course of a paper on photosynthetic induction, provides two results only which demonstrate an oxygen effect for *Scenedesmus*. At 20·5°C, high light intensity (10,000 lux, $aP = 10Rd$), and high CO_2 concentration (4 per cent., two-vessel method, plant in phosphate solution), he obtained a 43 per cent. increase in the rate of oxygen output (rP) by transfer from oxygen to nitrogen, the assimilation quotient remaining unchanged. Later in the same experiment the inhibition was only 11 per cent. and he states, "The influence of the partial pressure of oxygen varies with the internal conditions of the cell."

(vii).—Cladophora.—Our experiments on oxygen inhibition of photosynthesis were initiated by an exploratory series carried out by the senior author and Mr. (now Professor) G. E. Briggs, at Cambridge. The plant used was *Cladophora glomerata*.

Methods: The Warburg direct method was used, with four vessels, all illuminated from below by light reflected by a mirror from filament lamps of 100–500 W, each one opposite a vessel. As with more recent experiments described here, each gas mixture was equilibrated with the appropriate buffer solution before being passed through the manometer vessel.

The alga was gathered from the river Cam on the day prior to the experiment, cut into short branched pieces, and kept overnight in tap water. Branches carrying epiphytic algae were discarded. The activity was such that only a small number of filaments was required in each vessel and shading by overlapping was thus avoided. The shaking (100–120 oscillations/min) did not appear to damage the filaments.

The dry weight of the samples used varied from 6 to 25 mg. As it is difficult to obtain closely comparable samples of such a filamentous alga, each sample served as its own control. It was suspended in the buffer solution under a given atmosphere and readings were taken every 10 min until the rate became steady, or until a drift line was established. The atmosphere above the buffer was then changed; measurements were continued and usually the experiment was concluded after a second gas change, which restored the original conditions.

The main objection to this procedure is that the photosynthetic rate rarely remains constant for long, usually, but not always, drifting downwards. In early experiments we used a Warburg buffer (No. 9) made up in distilled water, and the rapid drift in the rate during the experimental period (2–3 hr) could be shown to be associated with reduction in the CO_2 concentration. The drifts were in part, however, due to other causes, for when buffer was made up in river water, the rate of downward drift was reduced. In most experiments it was possible to keep the basic rate fairly constant over a period of some hours, (1) by using less plant material in proportion to the volume of buffer (e.g. 6 mg/4 ml), and (2) by using 1 per cent. sodium bicarbonate made up in river water and filtered. This, when used, approximated in composition to Warburg buffer No. 11, but contained rather more bicarbonate. Calculations show that during experiments lasting 230–250 min, with a high rate of photosynthesis, the CO_2 concentration fell approximately from 350 to 90×10^{-6} mole/l, both of which are high concentrations, unlikely to limit the rate. In one experiment the rate in 100 per cent. oxygen remained almost constant over 6 hr.

Results: The following is a summary of the results obtained under conditions of high light intensity, (near saturation) and high CO_2 concentration ($>91\times10^{-6}$ mole/l, $Rd<aP/10$) (see Table 2):

When the concentration of oxygen in equilibrium with the aqueous medium surrounding *Cladophora* was decreased from 100 to 2·5 per cent. there was a consistently rapid and marked increase in *aP*. This effect persisted as long as the plant was maintained at the low concentration of oxygen, but was completely reversible. A gas change from 2·5 to 100 per cent. oxygen caused a rapid and marked decrease in *aP*.

TABLE 2

OXYGEN INHIBITION OF PHOTOSYNTHESIS AND RESPIRATION OF CLADOPHORA GLOMERATA

High CO_2 concentration ($>91\times10^{-6}$ mole/l); high light intensity; temperature 25°C

Expt. No.	100% Oxygen			2·5% Oxygen			Percentage Effects (based on higher of two rates)		
	Rd	*aP*	*rP*	*Rd*	*aP*	*rP*	*Rd*	*aP*	*rP*
C1A	4·4	52·5	56·9	3·9	72·5	76·4	−11	+28	+26
C7	2·3	11·5	13·8	1·9	21·0	22·9	−17	+45	+40

In six experiments, in each of which two closely matched samples of *Cladophora* were exposed, one to 2·5 per cent. oxygen, and the other to 100 per cent. oxygen, the differences in *aP* between paired samples were always large and in the same direction, i.e. the rate was higher in 2·5 per cent. oxygen.

In experiments with individual samples of *Cladophora*, the atmosphere over the plants was changed 57 times, either from 2·5 to 100 per cent. oxygen, or in the reverse direction. Oxygen inhibition was shown clearly in 56 of these exchanges, which were made thus:

(i) In 18 changes, from 100 to 2·5 per cent. oxygen, a marked rise in *aP* occurred, followed by a fall to the expected rate (allowing for time drifts) in 17 of the samples when 100 per cent. oxygen was reintroduced (see Figs. 3(*a*), 3(*b*), 4).

(ii) In five changes from 2·5 to 100 per cent. oxygen, a marked fall in *aP* occurred, followed by reversal on return of the plant to 2·5 per cent. oxygen (see Figs. 3(*c*), 3(*d*)).

(iii) In six simple changes, from 100 to 2·5 per cent. oxygen, all showed a marked rise in *aP*.

(iv) In five simple changes, from 2·5 to 100 per cent. oxygen, all showed a marked fall in *aP*.

The reversibility and the time-scale of the effect are shown in Figures 3 and 4. The interpolated lines joining the rates before and after the gas changes are drawn visually, and no claim is made as to any accuracy in their actual form. While the drifts of the rate with time make it difficult to be certain that the reversibility is always complete, experiments of the type shown in Figure 3 (expts. C13, C19) indicate complete reversibility. This is made clearer in Figure 4 (expts. C9, C8), where a control sample was kept in one gas throughout the experiment and showed a drift in rate parallel to that suggested for the treated plant.

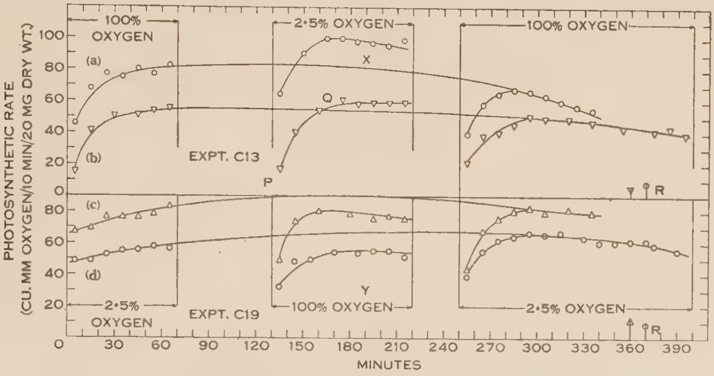

Fig. 3.—*Cladophora glomerata:* Drift of apparent photosynthetic rate with time, showing reversible inhibition of photosynthesis by high oxygen concentration. Temperature 25°C, $[CO_2] > 91 \times 10^{-6}$ mole/l, high light intensity (near saturation) for both experiments. *R*, mean respiration rates at conclusion of the experiments.

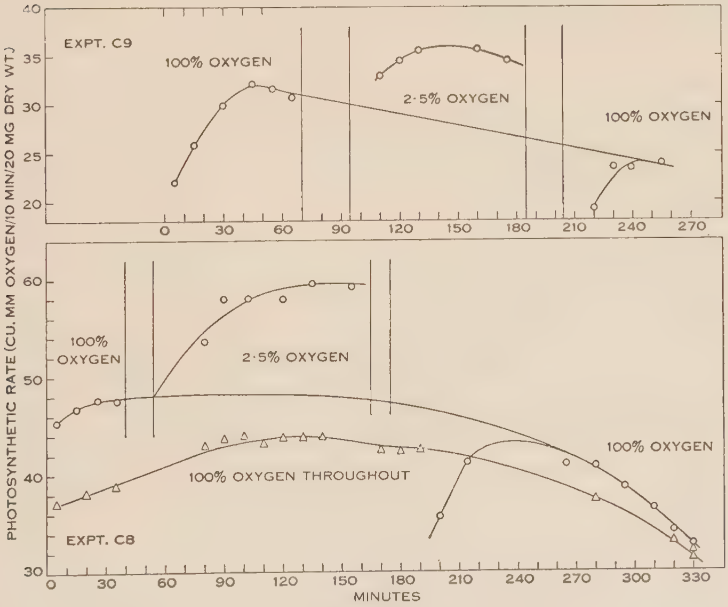

Fig. 4.—*Cladophora glomerata:* Drifts in apparent photosynthetic rates in oxygen with time with and without interpolation of periods in low oxygen concentration. Temperature 25°C; $[CO_2] > 91 \times 10^{-6}$ mole/l; high light intensity, near saturation.

The oxygen inhibition was large and (as Warburg (1920) noted) variable, a feature which is accentuated in this series of experiments because of a lack of uniformity in plant samples and experimental treatment. Taking expt. C13, Figure 3, as an example, we chose to compare the rates at time XY using the hypothetical drift lines and avoiding use of the initial lag phase (PQ) after the plant was transferred to a new atmosphere. For all experiments the percentage inhibition (based on the rate in low oxygen) ranged from 5 to 74 per cent. The mean was 30 per cent. and standard deviation 15·1.

Table 3

OXYGEN INHIBITION OF APPARENT PHOTOSYNTHETIC RATE IN MARINE ALGAE

Except where otherwise stated, all experiments were carried out at 20°C and high CO_2 concn. ($= 36\times10^{-6}$ mole/l)

Species	Light Source	Light Intensity (lux)	Changes in Oxygen Concn. (%)	Inhibition of *aP* (as % of rate in low oxygen concn.)	Reversal of Inhibition	Comments on Rate–Time Curves (see Fig. 5)
Green algae						
Enteromorpha	Sodium	17,000	20–100–20	18	+	Upward drift, then steady
Caulerpa scalpelliformis	Sodium	17,000	20–100–20	62	+	Downward drift
Cladophora sp.	Sodium	17,000	20–100–20	43	+	Rapid downward drift
Bryopsis sp.	Sodium	17,000	20–100–20	5	+	Rapid downward drift
Red algae						
Nitzemia sp.	Mercury	9,500	5–100–5	20	+	Downward drift
Phacelocarpus complanatus	Mercury	9,500	5–100–5	36	+	Rate steady
Chylocladia muelleri	Mercury	9,500	5–100–5	34	+	Rapid downward drift
Polysiphonia abscissa	Sodium	17,000	5–100–5	100,† 88	+	Rapid downward drift
Unidentified sp.	Mercury	9,500	5–100–5	48	+	Rapid downward drift
*Porphyridium cruentum**	Mercury	9,500	1–100–1	100†	+	Upward drift

* Temperature 25°C, $[CO_2] = 1\times10^{-6}$ mole/l.

† Oxygen uptake in the light in 100 per cent. oxygen.

In some experiments the oxygen effect on the dark respiration rate (*Rd*) was measured. Change of oxygen concentration from 2·5 to 100 per cent. increased *Rd* by as much as 20 per cent. However, as *Rd* itself was low in comparison with photosynthetic rate, the oxygen inhibition of photosynthesis cannot be explained in terms of the oxygen effect on the dark respiration (cf. Table 2 and Fig. 3). The same conclusion has been reached by those working with *Chlorella*.

(*b*) *Marine Algae*

In the following series of experiments the existence of a typical oxygen effect on photosynthesis was established for some marine algal species. The manometric technique was similar to that used for *Cladophora*. The algae were maintained in a photosynthetically-active condition for a few days in aerated sea-water, in the diffuse light of the laboratory. They were washed as free as possible of epiphytic organisms and were suspended, for the duration of each experiment, in the saline buffer solution E recommended by Emerson and Green (1934). This had an initial pH of 7·6 and a CO_2 concentration of 36×10^{-5} mole/l. All gases were equilibrated over the bicarbonate-carbonate buffers in saline solution before being passed into the vessels. For experiments with green algae, tubular sodium discharge lamps (Osram SD/H82) mounted horizontally under the glass window of the water-bath were used. For the red algae, mercury discharge lamps (Osram MA/H, 250 W) held vertically were used, the light being reflected upwards by a mirror. The bath temperature was controlled to ±0·04°C.

In all these experiments the light intensity and the CO_2 concentration were high but not saturating. For most species the rate of photosynthesis declined during the 3-hr course of the experiment; these drifts, however, appear to be due to factors other than the CO_2 concentration, and they were absent for some species with a high photosynthetic rate.

Results are summarized in Table 3 and some data are plotted in Figure 5. Oxygen effects are calculated from the curves (as for *Cladophora*) on the basis of the rate in low oxygen concentration. For all species used there was a clear and reversible depression of photosynthesis by high oxygen concentration. In two experiments the high oxygen concentration depressed the photosynthesis below the compensation point, there being gas uptake in the light. As respiration was not measured in these experiments we have recorded those data as 100 per cent. inhibition of the apparent photosynthesis rate.

(*c*) *Bryophyta and Pteridophyta*

The moss *Funaria hygrometrica* has proved particularly suitable for photosynthesis experiments by the Warburg direct method, aerial leaves being very thin and capable of survival for many hours in carbonate-bicarbonate buffers. The moss was grown on soil in a glasshouse, and before each experiment the plants in a small tuft were carefully separated and washed in the appropriate buffer solution. From each plant, pieces of the green leafy stem from 2 to 5 mm long were cut, blotted dry, and weighed out in groups. Each Warburg vessel contained 0·2–0·4 g fresh weight in 10 ml buffer and was illuminated from below with light from a sodium discharge lamp (Osram SD/H82).

Here we deal only with experiments at high light intensity (30,000–33,000 lux) and high CO_2 concentration. Typical experimental data are plotted in Figure 6. The dark respiration was measured at the beginning and the end of each experiment in whichever gas mixture was used for the initial and final light periods, but gas changes in the course of the experiment were made only for samples in the light.

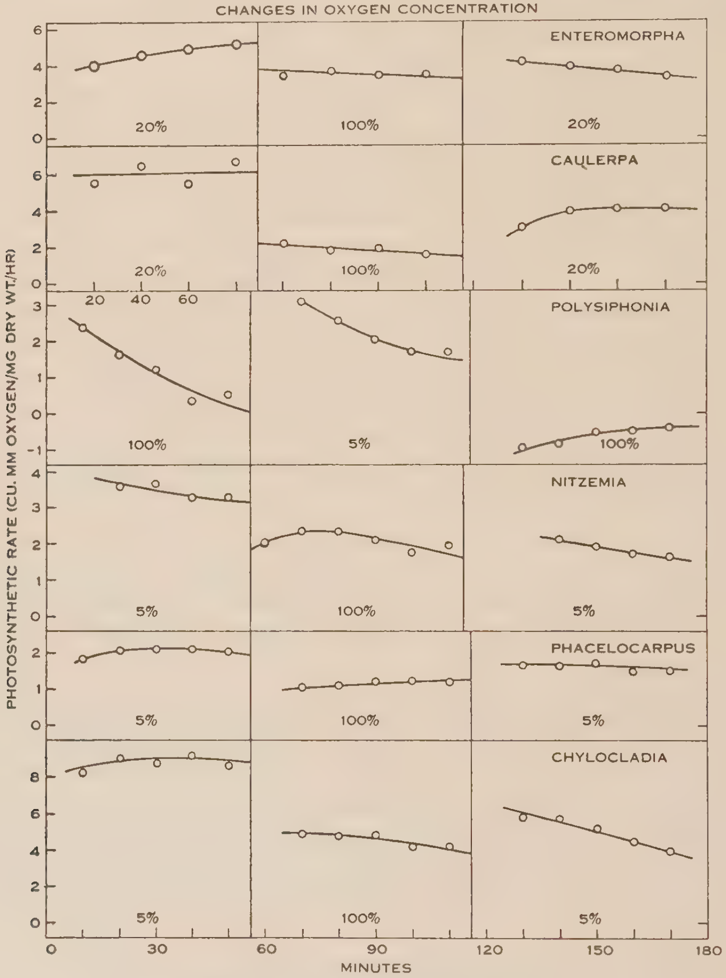

Fig. 5.—Oxygen inhibition of apparent photosynthesis in some marine algae. Details of each experiment will be found in Table 3.

On changing from low oxygen concentration (approximately 0·5 per cent.) to 100 per cent. oxygen there was a marked and reversible inhibition of *aP*. Judging from the respiration rates of two comparable samples in each gas, there was also a marked increase of respiration at the higher oxygen level, varying with time from 72 to 55 per cent. of the rate in oxygen. It is probable that the dark respiration rates are best given by the second set of dark readings at *BB′* (Fig. 6), after the low oxygen concentration has been raised by the evolution of oxygen in the light. Nevertheless, we have estimated *Rd* from the interpolated lines *AB*, *A'B'*. The effect of oxygen on the rate of true photosynthesis has then been estimated from these curves in two ways:

(i) We have assumed equality in leaf area of the paired samples and calculated percentage inhibitions of true photosynthesis from the paired samples at points *C*, *D*, *E* of the rate/time drift (Fig. 6). On the basis of the rate in low oxygen these are 14, 20·8, and 21·9 per cent. respectively.

(ii) We have also used the interpolated lines *YZ* and *Y'Z'* at time *D* to calculate the oxygen inhibition for each sample separately, thus obtaining two results for the whole experiment. The figures are 20 and 17 per cent. respectively, indicating complete reversibility within limits of experimental error.

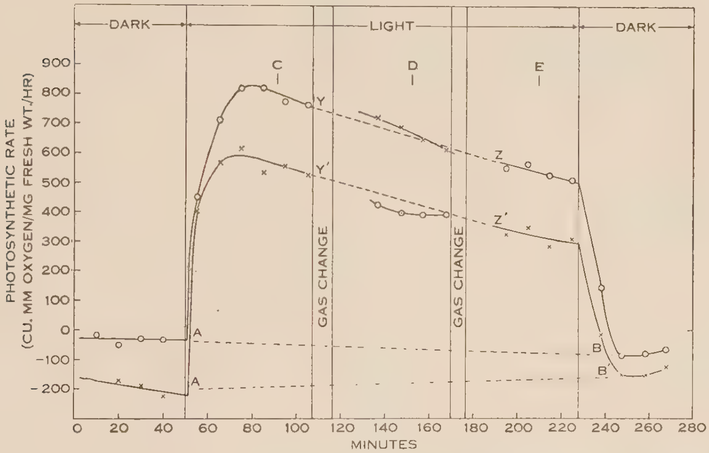

Fig. 6.—*Funaria hygrometrica:* Comparable samples initially in nitrogen (○) and oxygen (×) respiring in the dark, illuminated at 50 minutes. Gas phase reversed at YY' and again at ZZ'. Calculation of percentage inhibition as in the text. Temperature 25°C; $[CO_2] = 91 \times 10^{-6}$ mole/l; light intensity 30,000 lux.

As these percentage effects are calculated for the true rate of photosynthesis, it is clear that the inhibitory effect of oxygen on the photosynthesis is not explicable in terms of the effect of oxygen on the dark respiration.

Results of all experiments with *Funaria* are given in Table 4. As will be seen in the last line of this table, the effect of oxygen on photosynthesis at high CO_2 concentration is not significant over the range 5–20 per cent. oxygen.

In one experiment with a species of filmy fern (*Hymenophyllum*) it was possible to show a clear oxygen effect although the rate of photosynthesis drifted rapidly downwards reaching half its initial value in 4 hr. The fronds evidently suffered from immerson in bicarbonate-carbonate buffer, turning black if left in it for 12 hr. Thus at 25°C, with a light intensity of 6500 lux (sodium lamp), and a CO_2 concentration of 91×10^{-6} mole/l, and with gas changes from 20 to 100 to 20 per cent. oxygen, the oxygen inhibition of *aP* was 35 per cent. for this fern.

(*d*) *Angiosperms*

The only published record of the oxygen inhibition of photosynthesis in a higher plant is that of McAlister and Myers (1940); this is also the first record of the oxygen inhibition of CO_2 uptake. For young wheat leaves, at nearly saturating light intensity (50×10^4 ergs/cm²/sec) and low CO_2 concentration (0·03 per cent. in the gas phase), these authors found that the rate of CO_2 assimilation in air was 23 per cent. lower than that in 0·5 per cent. oxygen. The effects of higher oxygen concentrations were not studied.

We have found that high oxygen concentrations inhibit photosynthetic oxygen production in a wide variety of angiosperms.

Table 4

OXYGEN INHIBITION OF PHOTOSYNTHESIS IN FUNARIA HYGROMETRICA

In all these experiments the rate of photosynthesis drifted downwards over a period of 4 hr (see Fig. 6). Percentage effects based on the rate in the lower oxygen concentration. $[CO_2] = 91 \times 10^{-6}$ mole/l; light intensity (sodium lamp) = 30,000–33,000 lux; temperature 25°C

Changes in Oxygen Concentration (%)	Percentage Inhibition		Reverse of Inhibition
	aP	*rP*	
0*–100–0*	34	30	+
0*–100–0*	36	19	+
100–0*–100	33	15	+
100–0*–100	21	18	+
100–5†–100	25	16	+
5†–100–5†	29	26	+
5†–100	45	34	
5†–100	53	35	
5†–100	67	44	
20†–100–20†	90	74	+
20†–5–20†	7	0	

* Indicates hydrogen.

† The 5 and 20 per cent. oxygen mixtures contain nitrogen.

(i) *Aquatic species.*—A number of submerged or floating aquatic angiosperms are suitable for experiments by the Warburg direct method: the leaves are thin, the cuticle thin or absent, and they are not obviously harmed by immersion for periods of up to 6 hr in carbonate-bicarbonate buffers. In the submerged species the possible complications of stomatal control are absent.

Method: The Warburg direct method was used, portions of the leafy shoots being either immersed in the buffer (*Vallisneria*, *Cabomba*) or partly immersed, partly floating (*Callitriche*). For *Lemna*, whole fronds were used, floating on the surface. For *Nuphar*, circular disks were cut from mature leaves and a single disk was floated on the buffer in each vessel. For all species the photosynthetic rate drifted downwards over the experimental period, even at high CO_2 concentration; the rate of drift was least in *Cabomba*.

Results: At high light intensity and high CO_2 concentration there were clearly-marked oxygen effects between 20 and 100 per cent. oxygen, and in some cases smaller effects between 2·5 and 20 per cent. oxygen (Table 5). The inhibition of oxygen output by oxygen was reversible. For comparison with other species all oxygen effects are calculated on the basis of the rate in air, the + sign indicating inhibition by the higher of the two oxygen concentrations (Table 5). *Lemna* gave the only anomalous result (duplicated), an inhibition of oxygen output on passing from air to 5 per cent. oxygen, as well as the usual inhibition on passing from air to 100 per cent. oxygen. In this plant the frond was floating and uncut. Although it has been reported

Table 5

OXYGEN EFFECTS IN SOME AQUATIC ANGIOSPERMS

Sodium lamps were used for the light source except for *Cabomba caroliniana* when filament lamps were used. $[CO_2] = 91 \times 10^{-6}$ mole/l

Species	Temp. (°C)	Saturating Light Intensity (lux)	Changes in Oxygen Concn. (%)	Inhibition of aP† (as % of rate in lower oxygen concn.)	Reversal of Inhibition	Comments on Rate–Time Curves
Vallisneria spiralis	30	9,000	20–100–20	+23	+	Steady downward drift
			20–5–20	0		
Lemna minor	25	35,000	20–100–20	+58	+	Linear downward drift
			20–100–20	+52	+	
			20–5–20	−9		
			20–5–20	−19		
Nuphar sp.	25	35,000 (below satn.)	20–100–20	+43	+	Marked downward drift
Cabomba caroliniana	25		20–2·5–20	+16	+	Drift slight or absent
			20–5–20	+5	+	
			20–100–20	+47	+	
Callitriche sp.	30*	30,000	20–100–20	+18	+	Steady downward drift
			20–5–20	+16	+	
	25*	30,000	20–5–20	+16	+	Steady downward drift
			20–100	+34	+	
			20–100	+35	+	
			20–5	0		
			20–100	+33	+	
			2·5–20	+5	+	

* $Rd = aP/5$ at 30°C and $aP/7$ at 25°C.

† The + sign indicates inhibition by the higher of the two oxygen concentrations.

that the stomata of *Lemna* are immobile (Reuter 1948), it is possible, in view of Williams' work (1954), that the stomata close at low oxygen tension. This could affect the rate if CO_2 uptake is mainly from the gas phase.

(ii) *Land Plants.*—For exploratory experiments on the oxygen effect in the photosynthesis of land plants the continuous method is not convenient, if only by reason of the need for very careful matching of the CO_2 concentration in the two gas streams; or, alternatively, the need for using high CO_2 concentrations with consequent absorption problems. A modification of the manometric technique has proved suitable, although it has definite limitations. Portions of aerial leaves cannot usefully be immersed in carbonate-bicarbonate buffers, but, in spite of diffusion difficulties, data of value can be obtained if the leaf is illuminated directly in a closed vessel containing the shaken buffer solution in a separate compartment.

Methods: In this experiment, the Dickens-Simer manometric vessels were used (Dixon 1943). The buffer solution was placed in the outer rim and a leaf disk, approximately 1 in. in diameter, was placed flat with the abaxial surface downwards facing the source of light. The edges of the disk were moistened before each experiment, but injection of the air spaces with water was guarded against. The vessels were shaken at a speed of 100 excursions per minute.

The disadvantages of this technique are well known, and it would be useless for some types of study. At the beginning of illumination or change of gas phase the apparent photosynthetic rate is reduced because of the slow diffusion of the CO_2 from the buffer, as compared with its rapid uptake by the leaf. In preliminary studies it was not found possible to increase the rate of equilibration of the CO_2 between the buffer and the gas phase. When a bicarbonate solution (M/10 or M/1) is shaken with CO_2-free air in a closed manometer vessel, equilibration is reached in about 1 hr, the half-time being 10 min. These periods are not significantly altered by the addition to the buffer of glass beads, powdered glass, charcoal, colloidal ferric bicarbonate, or by exposing the buffer to the atmosphere on filter paper. No advance in the technique seems possible along these lines.

When a leaf disk of *Ampelopsis* is placed in the manometric vessel as described above, with M/10 bicarbonate in the rim, and shaken under high light intensity, the rate of increase of pressure in the vessel rises steadily to a maximum value reached after 1 hr; thereafter the rate remains constant in this species for several hours. In other species tried the high rate is reached much more quickly and subsequently drifts slowly downwards. If, at any time, the leaf disk is darkened, the pressure continues to rise but at a rapidly decreasing rate, until at about 1 hr after darkening an uptake of gas is recorded due to respiration. The half-time for the fall in darkness from the maximum rate to zero pressure change is about 10 min.

Under such conditions, we assume that the pressure increase during the illumination is the net effect of three processes: (1) the uptake of CO_2 by the leaf; (2) the equal output of oxygen by the leaf, assuming an assimilation quotient of 1·0; and (3) the output of CO_2 from the buffer solution. The increase in the rate is therefore simply due to the increase in the concentration gradient of CO_2 between air and buffer. When this is sufficiently great, however, (when the CO_2 may be limiting photosynthesis), we assume that rate (1) equals rate (3) and the pressure change in the steady state will be a measure of the oxygen output in photosynthesis. Even if maximum possible rates of photosynthesis cannot be measured by this method, owing to the diffusion lag, it should be possible to demonstrate oxygen inhibition of photosynthesis if such occurs. Its major defect is that conditions bringing about a change in the rate of photosynthesis must alter the CO_2 concentration in the gas phase. Thus oxygen inhibition so measured would not be maximal, as it is to be expected that after a fall in *aP* the CO_2 concentration in the gas phase would rise and, if limiting, increase the rate of *aP* again. The method is entirely unsuitable for the study of induction or of transitional effects but it has advantages of its own. The apparatus is very simple and it is possible to use two closely comparable small samples, one from each half of the same leaf. In our experiments these gave almost identical rates in the steady state, but differing from leaf to leaf.

TABLE 6

OXYGEN INHIBITION OF PHOTOSYNTHESIS IN SOME LAND PLANTS

Sodium lamps were used for the light source, the intensity being approx. 35,000 lux; CO_2 concentration unknown, probably limiting; temperature 30°C

Species	Buffer Used (in rim of vessel)	Changes in Oxygen Concn. (%)	Inhibition (as % of rate in lower oxygen concn.)		Reversal of Inhibition
			aP	*rP*	
Hydrangea hortensis	Warburg No. 11	5–20–5*	11		+
		5–20–5*	7		+
		5–100–5†	30		+
		5–100–5†	40		+
	Warburg No. 9	5–100	25		
		100–0‡–100	18		+
		0‡–100–0‡	19		+
		100–20–100	32	24	+
Nicotiana tabacum	Warburg No. 9	20–100–20	41		+
		20–5–20	0		
Ampelopsis veitschii	M/1 $KHCO_3$	20–100–20	64		−
		20–100–20	39		−
		20–100–20	41		+
		20–100–20	78		50%
		20–100–20	77		50%
		20–5–20	(−25)		−
		20–5–20	(−45)		Partial
		20–5–20	(−20)		Partial

*† Same disks used on successive days.

‡ Indicates nitrogen.

A test of the method was made with *Hymenophyllum* fronds. Some were submerged in the buffer (as described in Section II (*c*)), others were floated on a thin film of water and supplied with CO_2 from the buffer in the annular rim of the vessel. Closely similar curves for oxygen output were obtained and the percentage oxygen effect was 35 per cent. in both types of experiment.

Results: The results obtained by these means (Table 6) indicate that the oxygen effect on photosynthesis occurs in the aerial leaves of angiosperms. The results for *Ampelopsis* show the anomaly noted for *Lemna*, i.e. although the normal oxygen inhibition occurs on passage from air to 100 per cent. oxygen, there is also, in some experiments, an inhibition when the change is made from air to 5 per cent. oxygen. As these anomalous effects have only been found with organs carrying stomata, it is tempting to suggest that they are due to stomatal closure, in spite of the fact that the leaves used had circular cut edges.

In these higher plant leaves, after a period in oxygen, reversal of inhibition was not always obtained, but in such experiments it was noticed that the leaf disks showed signs of injection with water. It is proposed to investigate oxygen effects with the higher plants with more satisfactory techniques.

The results reported in this paper will be discussed in a second publication dealing with the effect of various factors on the oxygen inhibition of photosynthesis.

III. REFERENCES

BRIGGS, G. E., and WHITTINGHAM, C. P. (1952).—*New Phytol.* **51**: 236.

DIXON, M. (1943).—"Manometric Methods." 2nd Ed. p. 94. (Cambridge Univ. Press.)

EMERSON, R., and GREEN, L. (1934).—*J. Gen. Physiol.* **17**: 817.

GAFFRON, H. (1939).—*Cold Spr. Harb. Sym. Quant. Biol.* **7**: 377.

GAFFRON, H. (1940).—*Amer. J. Bot.* **27**: 204.

HILL, R., and WHITTINGHAM, C. P. (1953).—*New Phytol.* **52**: 133.

MCALISTER, E. D., and MYERS, J. (1940).—*Smithson. Misc. Coll.* **99** (Publ. No. 3591): 1.

MIYACHI, S., IZAWA, S., and TAMIYA, H. (1955).—*J. Biochem., Tokyo* **42**: 221.

MOYSE, A. (1953).—*C. R. Acad. Sci., Paris* **236**: 111.

REUTER, L. (1948).—*Phyton (Austria)* **1**: 76.

SURBECK, E., HOLT, V., and LUND, E. J. (1925).—*Proc. Soc. Exp. Biol., N.Y.* **23**: 681.

TAMIYA, H., and HUZISIGE, H. (1949).—*Acta Phytochim., Tokyo* **15**: 83.

WARBURG, O. (1920).—*Biochem. Z.* **103**: 188.

WASSINK, E. C., VERMEULEN, D., REMAN, G. H., and KATZ, E. (1938).—*Enzymologia* **5**: 100.

WILLIAMS, W. T. (1954).—*J. Exp. Bot.* **5**: 343.

GLUTAMINE AND ASPARAGINE AS NITROGEN SOURCES FOR THE GROWTH OF PLANT EMBRYOS *IN VITRO*: A COMPARATIVE STUDY OF 12 SPECIES

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Summary

Subsequent to earlier work on *Capsella bursa-pastoris* (L.) Moench. embryos of nine plant families, monocotyledons and dicotyledons, excised from young developing ovules were grown in a "sitting drop" culture.

For each species, the embryonic growth in length was compared in a control medium, i.e. without nitrogen, and in media containing L-glutamine and L-asparagine. In all cases the growth in glutamine was superior.

Additional data on *Capsella* are presented which deal with the growth-concentration relationships of glutamine and asparagine. Glutamine increasingly stimulates growth up to 600 mg/l. Asparagine stimulates growth at 10 mg/l but becomes inhibitory at higher concentrations.

The inhibition of growth with asparagine (400 mg/l) was also found in *Arabidopsis thaliana* (L.) Heynh. and *Reseda odorata* L. However, at this concentration, asparagine proved stimulating in *Sisymbrium orientale* L., *Cleome viscosa* L., *Medicago tribuloides* Desr., and *M. orbicularis* All., in *Anagallis arvensis* L., *Datura stramonium* L., *Chenopodium album* L., and *Allium cepa* L.

An earlier suggestion about the nature of asparagine-induced inhibition is partly revised.

I. INTRODUCTION

Evidence that glutamine plays a more important role than asparagine in the anabolic activities in higher plants has accumulated during the last decade (Steward and Thompson 1954). The present paper adds to this evidence and advocates the use of glutamine in a special type of plant tissue culture. It may be reiterated that the nitrogen nutrition of young embryos is exceptional, as they are apparently unable to utilize nitrate nitrogen. The evidence for this stems from *in vitro* growth studies of embryos excised from developing ovules in which the effect of different nitrogen sources was investigated.

Rijven (1952) has demonstrated that torpedo-shaped embryos of *Capsella bursa-pastoris* grown on a basal medium with nitrate as sole nitrogen source show a rapidly decreasing growth rate and that this was not due to exhaustion of a component of the medium. Nevertheless, under these conditions the nitrogen source was apparently limiting growth, for addition of amino acids greatly reduced this decline of the growth rate.

More direct information is available from an investigation on *Medicago orbicularis*. A comparison made between growth of embryos cultured on a basal medium containing either calcium nitrate or calcium chloride showed no difference in growth. However, these embryos again respond markedly to the addition of a number of amino acids to the medium (Rijven, unpublished data).

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Embryos of *C. bursa-pastoris* and *M. orbicularis* respond to ammonium compounds but much less than to a number of amino acids. For both species, glutamine added as sole nitrogen source to the medium has been found superior to any other amino acid tried, and also to glutamic acid added in nitrogen-equivalent amounts. Paris *et al.* (1953) have reported similar responses by *Datura stramonium*. They also noted that the presence or absence of nitrate in the basal medium does not affect growth.

In a previous study (Rijven 1955) on the *in vitro* growth of embryos of *Capsella bursa-pastoris*, a marked contrast was observed in growth of embryos supplied either with glutamine or asparagine as nitrogen source, viz. a stimulation by glutamine and a retardation by asparagine, both as compared with a control to which no organic nitrogen was added. Also, asparagine reduced the growth when added to media containing glutamine. In those experiments only one concentration of asparagine was used, viz. 400 mg/l. Additional data concerning growth-concentration relationships for glutamine and asparagine are presented here.

Though this work has revealed that asparagine stimulates growth slightly at 10 mg/l, the opposing effects of glutamine and asparagine at higher concentrations have been confirmed. This surprising result invoked interest in whether or not similar growth responses would be found with these amides in other plants.

The choice of species was partly determined by the desire to cover some of the old scheme "glutamine- and asparagine-families" of Schulze as listed by Chibnall (1939). At the same time the 12 species tried cover nine plant families belonging to seven orders, two of them monocotyledons. Special attention has been given to the Rhoeadales.

II. Methods and Materials

As previously described (Rijven 1952) our method is a rather short period test (1–4 days) of growth in length of embryos excised from ovules and explanted in a modified "sitting drop" culture technique. The culture cells contain up to 20 embryos which are suspended in separate drops (about 30 μl) of liquid medium between two glass slides, to enable the measurement of their lengths by means of a microscope fitted with an eyepiece micrometer. Handling of the drops and of the embryos is done with sterilized braking-pipettes which were first introduced into the field of enzymic ultramicroanalysis by Linderstrøm Lang and Holter (1941). It may be stressed that these implements proved very useful. To allow heat sterilization, the capillaries were fitted with a high melting resin ("Araldite", from CIBA, Basle) instead of paraffin.

Previous results have shown that the growth of young plant embryos is very much affected by the quantity and quality of the nitrogen source. In *C. bursa-pastoris* a concentration of 400 mg/l of glutamine gives growth that is close to the maximal response. Lower concentrations limit the growth. This concentration of L-glutamine has therefore been adopted for experiments on embryos of other species. The same (*c.* equimolar) concentration is used in the L-asparagine series (asparagine is weighed as L-asparagine monohydrate; both amides were products of L. Light & Co. Ltd., England). In some cases other concentrations are used as well. For each species also a series with basal medium only, i.e. without a nitrogen compound added, was used as a control. However, except for the experiments on *Capsella* a minute amount of ammonium molybdate was present in the basal medium.

The basal medium consisted of 1 l. glass-distilled water to which was added 120 g sucrose, and as salt nutrients K_2HPO_4, 87 mg, KH_2PO_4, 340 mg, $CaCl_2$, 55 mg, $MgSO_4{\cdot}7H_2O$, 49 mg, $MnSO_4$, 0·45 mg, H_3BO_3, 0·062 mg, $ZnSO_4{\cdot}7H_2O$, 0·287 mg, $CuSO_4{\cdot}5H_2O$, 0·024 mg, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0·12 mg, ferric citrate, 15 mg, were added. The media, with pH $6{\cdot}0 \pm 0{\cdot}2$, were sterilized by filtration through sintered-glass filters.

The cultures were incubated at $26 \pm 0{\cdot}2$°C in a dark incubator and measurements made at 24-hr intervals.

The following species were tested in the order shown:

Medicago tribuloides Desr., December 1953, from Dickson Experimental Station, A.C.T.
Medicago orbicularis All., May 1954, from glass-house cultures
Anagallis arvensis L., red variety, March 1954, from roadside
Datura stramonium L., April 1954, from glass-house cultures
Chenopodium album L., May 1954, from roadside
Hordeum vulgare L., smooth awns, January 1955, from glass-house cultures
Allium cepa L., January 1955, from garden
Arabidopsis thaliana (L.) Heynh., March 1955, from glass-house cultures
Reseda odorata L., April 1955, from garden
Cleome viscosa L., October 1955, from glass-house cultures
Sisymbrium orientale L., November 1955, from roadside
Capsella bursa-pastoris (L.) Moench., November, December 1955, from roadside.

In general, up to 20 torpedo-shaped embryos between 0·5–1 mm in length were taken as initial material for each treatment.

The collection and dissection of the necessary number of ovules sometimes takes several hours so that embryos are not all treated in exactly the same way. This necessitates randomizing in the control medium in which the embryos are also dissected, prior to distribution over the treatments.

In such experiments it is unavoidable that the starting material, the excised embryos, vary in initial length. In general, the data for any one species was found to fit the formula:

$$y = a + bx, \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(1)$$

where y = length increment, and x = initial length. The regression of length increment on initial length is not the same in different species or often in different treatments.

In statistical analysis and in presentation of the data a pooled regression slope (regression coefficient b) has been used for each experiment except when the separate slopes of the different treatments were significantly different. Differences between y values, estimated at the mean of all x values, and found significant at the 5 per cent. level at least, are recorded in the final columns of the tables.

The treatments were not replicated in sets of culture cells, but in most cases the main issue of the experiments has been checked by repeating them, e.g. where unexpected results were obtained.

III. Results

The data on the Rhoeadales are presented prior to those of other plant groups; the latter follow in the chronological sequence in which the experiments were done as indicated in Section II.

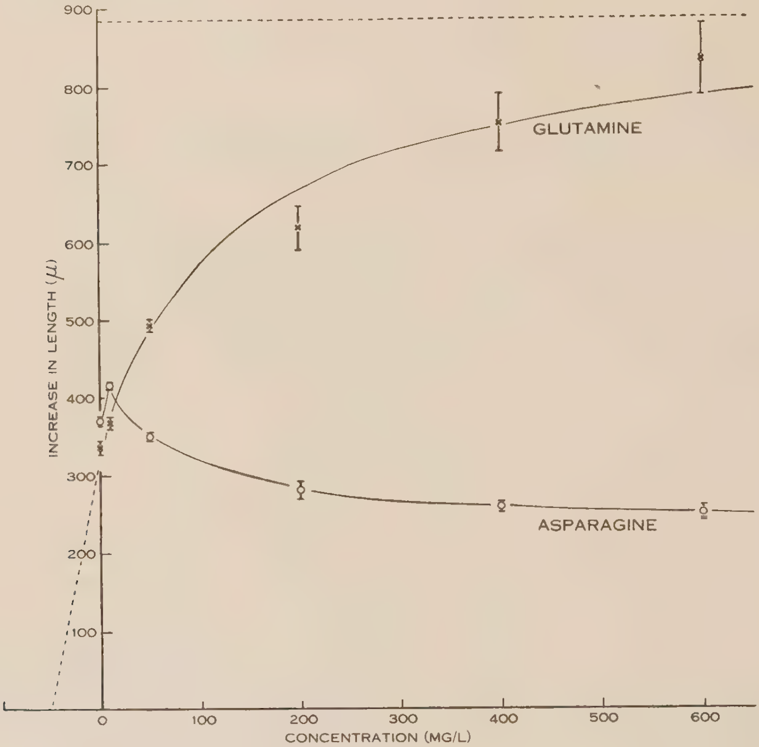

Fig. 1.—*Capsella bursa-pastoris.* Relation between growth value of embryos of 623·3 μ after 48 hr and various concentrations of glutamine and asparagine. The line drawn for glutamine, based on Table 1, represents the theoretical function $y = (884{\cdot}1c' + 44116{\cdot}59)/(c' + 131{\cdot}14)$, which is described more fully in Section IV. The line drawn for asparagine, based on Table 2, is empirical. The vertical lines at the dots represent the standard errors.

(*a*) Capsella bursa-pastoris, Arabidopsis thaliana, *and* Sisymbrium orientale

(i) Capsella bursa-pastoris.—In earlier work (Rijven 1955) the concentration of glutamine and asparagine used was frequently 400 mg/l. The choice of this concentration was based mainly on previous experience with different amino acids for which the effect of concentration had been tested (Rijven 1952). However, since the discovery of the contrasting effects of glutamine and asparagine, information on the effect of different concentrations of these particular amino acids has been lacking. A summary of some experiments to fill this gap is presented in Figure 1 and Tables 1 and 2.

Glutamine, as was expected, is progressively growth-promoting up to the highest concentration tried. In the mathematical treatment in Section IV the data of Table 1 have been shown to fit the Michaelis-Menten formula. In Figure 1 the curve drawn for glutamine represents the results of those calculations.

TABLE 1

EFFECT OF GLUTAMINE CONCENTRATION ON GROWTH OF EMBRYOS OF CAPSELLA BURSA-PASTORIS

Corrected estimates of length increment (y) of embryos at the mean initial length (x) of 623·3 μ cultured for 48 hr in basal medium (control) and in five media to which glutamine was added. The regression coefficient denotes the regression of length increment on initial length. The statistical treatment of the data in this and all succeeding tables is explained in the text

Treatment No.	Glutamine Concn. (mg/l)	No. of Observations	Regression Coefficient	y in μ at $x = 623{\cdot}3\ \mu$	Treatments Differing Significantly from the Particular Treatment
1	Control	17	0·324	337·2	*1*<2, 3, 4, 5, 6
2	10	15	0·282	369·0	1<*2*<3, 4, 5, 6
3	50	15	0·339	491·3	1, 2<*3*<4, 5, 6
4	200	13	0·446	615·5	1, 2, 3<*4*<5, 6
5	400	17	1·027	751·9	1, 2, 3, 4<*5*
6	600	13	2·739	830·7	1, 2, 3, 4<*6*

TABLE 2

EFFECT OF ASPARAGINE CONCENTRATION ON GROWTH OF EMBRYOS OF CAPSELLA BURSA-PASTORIS

Corrected estimates of length increment (y) of embryos at the initial length (x) of 623·3 μ cultured for 48 hr in basal medium (control) and in five media to which asparagine was added. The mean initial length was 632·6 μ. The regression coefficient denotes the regression of length increment on initial length

Treatment No.	Asparagine Concn. (mg/l)	No. of Observations	Regression Coefficient	y in μ at $x = 623{\cdot}3\ \mu$	Treatments Differing Significantly from the Particular Treatment
1	Control	9	0·266	369·9	2>*1*>4, 5, 6
2	10	15	0·266	414·3	*2*>1, 3, 4, 5, 6
3	50	14	0·266	350·3	2>*3*>4, 5, 6
4	200	14	0·266	281·9	1, 2, 3>*4*
5	400	18	0·266	259·7	1, 2, 3>*5*
6	600	9	0·266	251·4	1, 2, 3>*6*

Asparagine is stimulating at 10 mg/l, but, as concentration increases, becomes inhibitory, growth rate falling off to a steady level. The stimulation at 10 mg/l was not expected. The consequence of this finding, which was confirmed in two more experiments, is that an earlier attempt to explain asparagine-induced inhibition has to be revised (see Section IV). Nevertheless, the contrasting behaviour of *Capsella* embryos at higher concentrations of the homologous amides remains a peculiar phenomenon.

TABLE 3

EFFECTS OF ASPARAGINE AND GLUTAMINE ON GROWTH OF EMBRYOS OF ARABIDOPSIS THALIANA

Corrected estimates of length increment (y) of embryos at the mean initial length (x) of 285·8 μ cultured for 48 hr in basal medium (control) and in media containing asparagine or glutamine. The regression coefficient denotes the regression of length increment on initial length

Treatment No.	Treatment	No. of Observations	Regression Coefficient	y in μ at $x = 285{\cdot}8\ \mu$	Treatments Differing Significantly from the Particular Treatment
1	Control	15	0·472	190·1	2<*1*<3
2	Asparagine, 400 mg/l	10	0·472	128·5	*2*<1, 3
3	Glutamine, 400 mg/l	7	0·472	366·1	2, 1<*3*

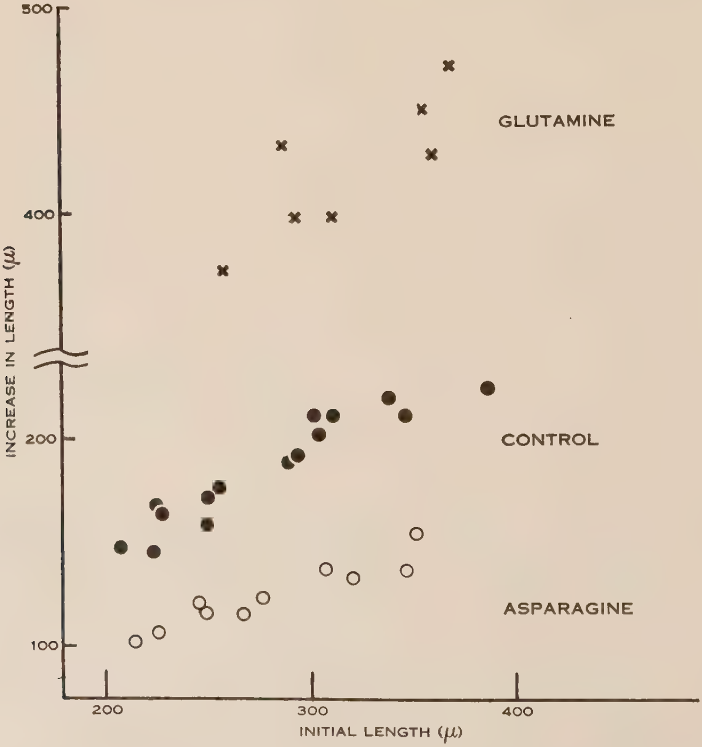

Fig. 2.—*Arabidopsis thaliana.* Dot diagram of observations on individual growth values after 48 hr and initial lengths of embryos grown in control media and in glutamine (400 mg/l) and asparagine (400 mg/l).

(ii) Arabidopsis thaliana.—This species was chosen in order to discover whether the results obtained on *C. bursa-pastoris* would be valid for other Cruciferae.

A. thaliana produces 30–40 ovules per ovary with embryos of about equal size. Dissection is easy. Torpedo-shaped embryos cover a range from 200–450 μ. Actually this small size is an advantage in our culture drop technique. The fact that flowering plants are easily obtained all year round together with the other properties makes this species a promising test object for more thorough studies.

The results obtained in a number of experiments support the expectations. Glutamine is strongly stimulating, but the interesting point is the inhibition found in the asparagine series when compared with the control series (Table 3 and Fig. 2). This result is in good agreement with the experience on *C. bursa-pastoris*.

(iii) Sisymbrium orientale.—Not all Cruciferae follow the same response pattern as *Capsella* and *Arabidopsis*, for in the case of *S. orientale*, a definite stimulation by asparagine was found, increasing with increasing concentration up to an optimal value of 200 mg/l (Table 4).

TABLE 4

EFFECT OF ASPARAGINE CONCENTRATION ON GROWTH OF EMBRYOS OF SISYMBRIUM ORIENTALE

Corrected estimates of length increment (y) of embryos at the mean initial length (x) of 552·7 μ cultured for 48 hr in basal medium and in four media containing different asparagine concentrations. The regression coefficient denotes the regression of length increment on initial length

Treatment No.	Asparagine Concn. (mg/l)	No. of Observations	Regression Coefficient	y in μ at $x = 552{\cdot}7$ μ	Treatments Differing Significantly from the Particular Treatment
1	Control	19	0·608	321·3	*1*<2, 3, 4, 5
2	10	13	0·746	361·9	1<*2*<4, 5
3	50	14	0·548	387·6	1<*3*<4, 5
4	200	16	1·070	496·5	1, 2, 3, 5<*4*
5	400	20	0·958	452·0	1, 2, 3<*5*<4

This result was not due to an irregularity in the manipulation or to an impurity in the asparagine used, for in another experiment in which *Sisymbrium* together with *Capsella* embryos were tested with the same medium, the findings for both were completely consistent with earlier results.

(*b*) Reseda odorata

This species belongs to the Resedaceae and as this family is claimed to be closely related to the Cruciferae, it was thought that asparagine-induced inhibition of embryo growth was likely to occur here also.

As a technical difficulty it should be mentioned that the integument of the ovules in the required young stages is already considerably lignified so that for the excision of embryos extra care is needed to prevent damage.

Glutamine again proved strongly stimulating. In five out of six experiments a significant inhibition by asparagine was found (Table 5). The inhibition was most evident during the first 3 days of culture. In some experiments longer incubation led to a recovery from the inhibition and in two experiments there was evidence for stimulation after 6 days. In one of these no initial inhibition was found. Attempts to reproduce this "adaptive behaviour" failed.

Table 5

EFFECT OF ASPARAGINE ON GROWTH OF EMBRYOS OF RESEDA ODORATA

Corrected estimates of length increment (y) of embryos at the mean initial length (x) of 486·4 μ cultured for 48 hr in basal medium (control) and in medium containing asparagine. The regression coefficient denotes the regression of length increment on initial length

Treatment No.	Asparagine Concn. (mg/l)	No. of Observations	Regression Coefficient	y in μ at x = 486·4 μ	Treatments Differing Significantly from the Particular Treatment
1	Control	31	0·368	288·6	2<*1*
2	400	16	0·368	212·9	*2*<1

(*c*) Cleome viscosa

This species of the Capparidaceae was tested for the same reason as *R. odorata*, viz. to check if the asparagine-induced inhibition could be found in more families of the Rhoeadales.

Table 6

EFFECTS OF ASPARAGINE AND GLUTAMINE ON GROWTH OF EMBRYOS OF CLEOME VISCOSA

Corrected estimates of length increment (y) of embryos at the mean initial length (x) of 614·1 μ cultured for 48 hr in basal medium (control) and in three media containing asparagine or glutamine. The regression coefficient denotes the regression of length increment on initial length

Treatment No.	Treatment	No. of Observations	Regression Coefficient	y in μ at x = 614·1 μ	Treatments Differing Significantly from the Particular Treatment
1	Control	16	0·032	293·4	*1*<2, 3, 4
2	Asparagine, 400 mg/l	20	0·793	378·4	1<*2*<3, 4
3	Asparagine, 600 mg/l	12	0·420	451·5	1, 2<*3*<4
4	Glutamine, 400 mg/l	16	0·149	980·5	1, 2, 3<*4*

The ovules and embryos of *C. viscosa* are very much like those of *R. odorata*; in dissection, therefore, the same precautions against damage are necessary.

Glutamine again proved strongly stimulating. Asparagine also was repeatedly found significantly stimulating though much less so (Table 6). This stimulation in *Cleome* is in contrast with the inhibition in *Reseda* notwithstanding the fact that the ovules appeared so much alike.

(*d*) Medicago tribuloides *and* M. orbicularis

These species, belonging to the Leguminosae, were chosen partly as representing the classical "asparagine type" of plant. The characteristic helical pods do not contain many ovules. Isolation proved difficult with *M. tribuloides*, but was easier with *M. orbicularis*, so this species was used more in the later stages. As Table 7 shows, the growth responses of *M. tribuloides* to the three treatments are well differentiated after 48 hr incubation, and these results lead to the conclusion that in this species glutamine induces more growth than asparagine, and the latter more than the control.

TABLE 7

EFFECTS OF ASPARAGINE AND GLUTAMINE ON GROWTH OF EMBRYOS OF MEDICAGO TRIBULOIDES

Corrected estimates of length increment (y) of embryos at the mean initial length (x) of 637·3 μ cultured for 48 hr in basal medium (control) and in media containing asparagine or glutamine, The regression coefficient denotes the regression of length increment on initial length

Treatment No.	Treatment	No. of Observations	Regression Coefficient	y in μ at $x = 637{\cdot}3\ \mu$	Treatments Differing Significantly from the Particular Treatment
1	Control	14	0·355	291·8	*1*<2, 3
2	Asparagine, 400 mg/l	19	0·480	401·9	1<*2*<3
3	Glutamine, 400 mg/l	17	0·609	518·4	1, 2<*3*

TABLE 8

EFFECTS OF ASPARAGINE AND GLUTAMINE ON GROWTH OF EMBRYOS OF MEDICAGO ORBICULARIS

Corrected estimates of length increment (y) of embryos at the mean initial length (x) of 631·0 μ cultured for 48 hr in basal medium (control) and in five media containing asparagine or glutamine or both. The regression coefficient denotes the regression of length increment on initial length

Treatment No.	Treatment	No. of Observations	Regression Coefficient	y in μ at $x = 631{\cdot}0\ \mu$	Treatments Differing Significantly from the Particular Treatment*
1	Control	5	0·108	240·9	*1*<2, 3, 4, 5, 6
2	Glutamine, 400 mg/l	15	0·795	535·9	1, 3<*2*<6
3	Asparagine, 400 mg/l	15	0·411	358·8	1<*3*<2, 4, 5, 6
4	Asparagine, 2000 mg/l	18	0·683	540·3	1, 3<*4*<6
5	Glutamine, 400 mg/l, + asparagine, 400 mg/l	16	0·775	571·8	1, 3<*5*<6
6	Glutamine, 400 mg/l, + asparagine, 2000 mg/l	16	0·850	630·1	1, 2, 3, 4, 5<*6*

* Treatments 2, 4, and 5 do not differ significantly.

Essentially the same results were repeatedly found for *M. orbicularis*. When a series containing glutamine and asparagine together (total nitrogen concentration thereby doubled) was included in the experiment beside the other series, the response of this series was not significantly different from that given by glutamine alone (see Table 8). Asparagine does not interfere with glutamine utilization as it actually does at this concentration in some Cruciferae. The effect of asparagine alone can be increased by increasing its concentration up to 2000 mg/l. The result of this treatment (No. 4 of Table 8) again is not significantly different from glutamine at a concentration of 400 mg/l. When, however, the amides were added together in these concentrations (treatment No. 6 of Table 8) the growth response was higher than that resulting from each alone.

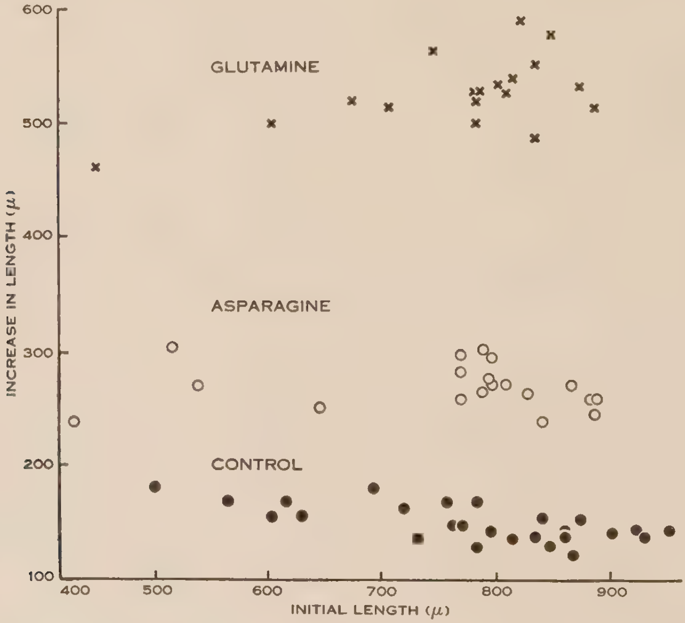

Fig. 3.—*Anagallis arvensis*. Dot diagram of observations on individual growth values after 48 hr and initial lengths of embryos grown in control media and in glutamine (400 mg/l) and in asparagine (400 mg/l).

(*e*) Anagallis arvensis

In *A. arvensis* (Primulaceae) the embryos remain straight and torpedo-shaped even in the mature seed. The excision of embryos from the ovules proved difficult, since the embryos were surrounded by a thick milky endosperm, which adhered to their surface.

The absence of correlation between growth and initial length which we have observed only in this species is noteworthy (see Fig. 3). This absence of significant correlation could mean that in these torpedo-shaped embryos growth is already restricted to certain well-defined meristematic zones which produce new, non-dividing cells at a constant rate. Therefore, in this species, it was sufficient to calculate the mean length increments and the standard errors (see Table 9). The same situation as in the medics is found here, viz. glutamine affording more growth than asparagine and the latter being stimulative also. Addition of both amides together resulted in a growth response not significantly different from that given by glutamine alone.

TABLE 9

EFFECTS OF ASPARAGINE AND GLUTAMINE ON GROWTH OF EMBRYOS OF ANAGALLIS ARVENSIS

Mean length increment of embryos grown for 48 hr in basal medium (control) and in media containing asparagine or glutamine or both

Treatment No.	Treatment	No. of Observations	Mean Initial Length (μ)	Mean Length Increment (μ)	Treatments Differing Significantly from the Particular Treatment
1	Control	18	601·6	167·6 (S.E. 5·4)	*1*<2, 3, 4
2	Asparagine, 400 mg/l	18	551·7	293·6 (S.E. 6·1)	1<*2*<3, 4
3	Glutamine, 400 mg/l	12	602·9	478·8 (S.E. 10·6)	1, 2<*3*
4	Glutamine, 400 mg/l, + asparagine, 400 mg/l	18	560·6	484·2 (S.E. 10·1)	1, 2<*4*

(*f*) Datura stramonium

This species, belonging to the Solanaceae, has been the object of extensive studies which are reviewed by Rappaport (1954). Testing of the relevant amides on this species has already been made by Paris *et al.* (1953). However, their culture method differs considerably from ours and their observations concern a longer period of incubation (8 days). These authors state that L-glutamine has a favourable effect on young embryos, while the effect of L-asparagine is only limited.

The results of one of our experiments are summarized in Table 10. Again glutamine stimulates most, but asparagine is also certainly stimulating on the growth of these small embryos, which is in good agreement with the results of the forementioned authors. In another experiment, asparagine and glutamine were added together; there was no discernible difference of growth stimulation as compared with glutamine alone.

Rietsema, Satina, and Blakeslee (1953) determined 8 per cent. sucrose to be optimal for small embryos. In contrast to the basal medium applied in the other species, 10 per cent. sucrose was used here, and in the second experiment described 8 per cent. was used. In both cases the growth rate was very high with four- to five-fold increases of length in 2 days for embryos initially 250 μ in length.

(*g*) Chenopodium album

This species belongs to the Chenopodiaceae which is listed by Chibnall (1939) as one of Schulze's "glutamine" families.

Collecting of embryos of the right stage is rather laborious as the ovaries contain only one ovule each. The young torpedoes are only slightly curved, but in measuring they are assumed to be straight, i.e. the length of the straight line connecting hypocotyl tip and cotyledonary tip was measured.

As Table 11 shows, the growth after 24 hr is already sufficient to demonstrate that glutamine stimulates growth more than asparagine although asparagine stimulates also. Especially in the glutamine series the correlation between growth and initial length is again good.

Table 10

EFFECTS OF ASPARAGINE AND GLUTAMINE ON GROWTH OF EMBRYOS OF DATURA STRAMONIUM

Corrected estimates of length increment (y) of embryos at the mean initial length (x) of 365·5 μ cultured for 48 hr in basal medium (control) and in media containing asparagine or glutamine. The regression coefficient denotes the regression of length increment on initial length

Treatment No.	Treatment	No. of Observations	Regression Coefficient	y in μ at $x = 365{\cdot}7\ \mu$	Treatments Differing Significantly from the Particular Treatment
1	Control	18	0·659	355·1	*1*<2, 3
2	Asparagine, 400 mg/l	16	0·659	618·8	1<*2*<3
3	Glutamine, 400 mg/l	15	0·659	924·9	1, 2<*3*

(*h*) Hordeum vulgare

H. vulgare was chosen as a representative of the Gramineae. The material was obtained from a single plant which, 2 months earlier, had been raised by explanting the immature embryo on an agar medium.

Table 11

EFFECTS OF ASPARAGINE AND GLUTAMINE ON GROWTH OF EMBRYOS OF CHENOPODIUM ALBUM

Corrected estimates of length increments (y) of embryos at the mean initial length (x) of 478·7 μ cultured for 24 hr in basal medium (control) and in media containing asparagine or glutamine. The regression coefficient denotes the regression of length increment on initial length

Treatment No.	Treatment	No. of Observations	Regression Coefficient	y in μ at $x = 478{\cdot}7\ \mu$	Treatments Differing Significantly from the Particular Treatment
1	Control	13	0·163	188·2	*1*<2, 3
2	Asparagine, 400 mg/l	10	0·486	236·8	1<*2*<3
3	Glutamine, 400 mg/l	10	0·659	376·3	1, 2<*3*

To calculate an index for growth in this species, which is lacking a "torpedo" stage, the long axes of the scutella were measured. Measurement *in situ*, i.e. in the culture drop, was not possible in all cases, as the embryos adhere strongly to menisci. These circumstances account for high variability, and, in this species alone, statistical treatment did not result in a significant difference between the asparagine and control series. Glutamine, however, proved significantly stimulating (see Table 12), and superior to asparagine.

The beneficial effect of asparagine on the growth of immature embryos of another gramineous plant, viz. corn, has been demonstrated by Haagen Smit, Siu, and Wilson (1945). It is quite certain now that addition of glutamine instead of asparagine will result in more vigorous growth.

TABLE 12

EFFECTS OF ASPARAGINE AND GLUTAMINE ON GROWTH OF EMBRYOS OF HORDEUM VULGARE

Corrected estimates of length increment (y) of embryos at the mean initial length (x) of 834·6 μ cultured for 72 hr in basal medium (control) and in media containing asparagine or glutamine. The regression coefficient denotes the regression of length increment on initial length

Treatment No.	Treatment	No. of Observations	Regression Coefficient	y in μ at $x = 834{\cdot}6\ \mu$	Treatments Differing Significantly from the Particular Treatment
1	Control	7	0·431	417·3	*1*<3
2	Asparagine, 400 mg/l	6	0·431	527·4	*2*<3
3	Glutamine, 400 mg/l	5	0·431	727·1	1, 2<*3*

(*i*) Allium cepa

A. cepa (Liliaceae) produces torpedo-shaped embryos; the one cylindrical cotyledon occupies the major part in the length. The results of one experiment are shown in Table 13. The three treatments are significantly different from each other. The glutamine series again does best, then the asparagine series.

TABLE 13

EFFECTS OF ASPARAGINE AND GLUTAMINE ON GROWTH OF EMBRYOS OF ALLIUM CEPA

Corrected estimates of length increments (y) of embryos at the mean initial length (x) of 750·1 μ cultured for 48 hr in basal medium (control) and in media containing asparagine or glutamine. The regression coefficient denotes the regression of length increment on initial length

Treatment No.	Treatment	No. of Observations	Regression Coefficient	y in μ at $x = 750{\cdot}1\ \mu$	Treatments Differing Significantly from the Particular Treatment
1	Control	9	0·410	335·4	*1*<2, 3
2	Asparagine, 400 mg/l	9	0·371	492·8	1<*2*<3
3	Glutamine, 400 mg/l	9	1·260	846·1	1, 2<*3*

IV. DISCUSSION

The general outcome of the experiments may be summarized by means of a histogram (Fig. 4) which shows the general beneficial effect of glutamine and asparagine when applied to the medium of young embryos *in vitro* at a concentration of

400 mg/l. Within eight orders of angiosperms no exception to this rule is found for glutamine; for asparagine exceptions have been found in certain members of the Rhoeadales.

Further, glutamine is shown to enhance embryonic growth considerably more than asparagine in all cases. This result is considered in excellent agreement with recent biochemical literature which ascribes to glutamic acid and glutamine an important primary role in plant nitrogen metabolism, and considers accumulation of asparagine rather the result of catabolic processes (Steward and Thompson 1954). This also holds for the Leguminosae (Meiss 1952); our results on the medics show that in this family also glutamine stimulates growth more than asparagine.

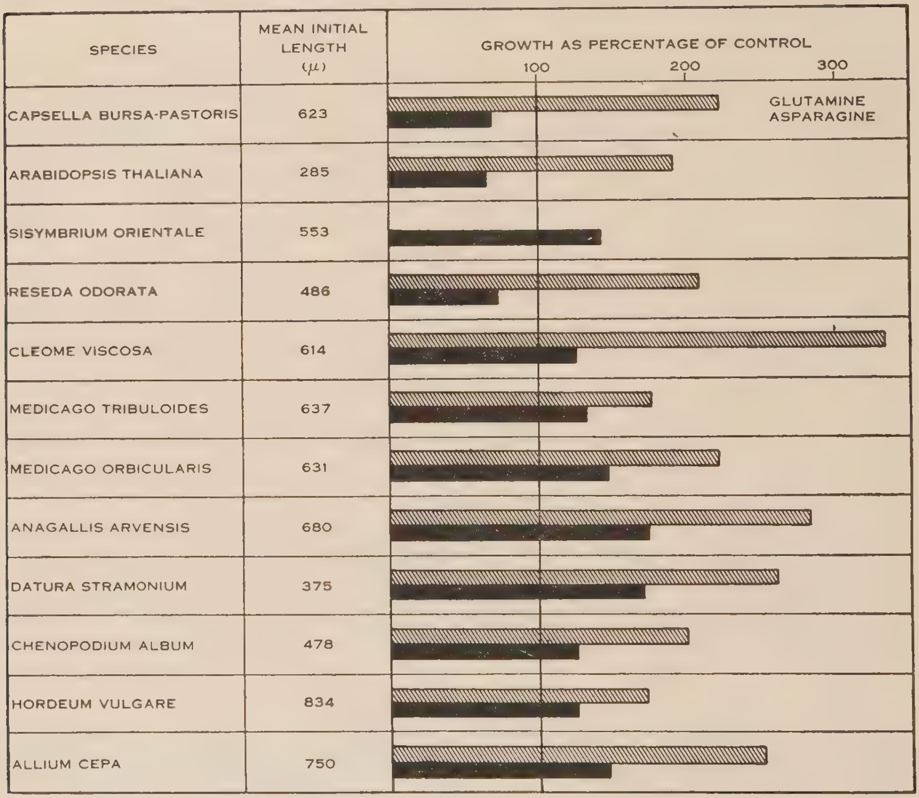

Fig. 4.—Histogram summarizing the effects of glutamine (400 mg/l) and asparagine (400 mg/l) on embryos of 12 species expressed as percentage of the growth in the control. The comparisons are made for 48 hr of culture, except for *Chenopodium album* (24 hr) and *Hordeum vulgare* (72 hr).

Glutamine, however, is not claimed to be superior to all amino acid mixtures, though this seems to be the case for "torpedoes" in *C. bursa-pastoris* (Rijven 1955).

Asparagine-induced inhibition of embryo growth can no longer be considered unique to *C. bursa-pastoris*. On the other hand, it was not possible to link the phenomenon as a taxonomic feature with Cruciferae or Rhoeadales though it has been observed so far only in this order. It certainly does not occur in *Chenopodium album* which represents another one of Schulze's "glutamine plants" (Chibnall 1939).

It is interesting to note the enormous contrast between *C. bursa-pastoris* and *Medicago orbicularis*. In the latter, asparagine stimulates progressively up to 2000 mg/l while in the former it becomes inhibitory at concentrations above 50 mg/l.

In all those cases where asparagine stimulates growth, it does not interfere with glutamine utilization. This is brought out by experiments on *M. orbicularis*, *Anagallis arvensis*, and *Datura stramonium*. On the other hand, in *Arabidopsis thaliana*, as in *C. bursa-pastoris*, asparagine reduced the embryonic growth in a medium which also contained glutamine.

In a previous attempt to explain the asparagine-induced inhibition in *C. bursa-pastoris*, it was suggested that asparagine may block competitively an enzyme system which turns glutamine-N into protein-N (Rijven 1955). In this connection it was thought that specific transpeptidases or transaminases or both might be involved. For this hypothesis two main arguments could be advanced: first, the compounds are homologues and affect the growth in opposite ways, and second, glutamine reverses asparagine-induced inhibition while asparagine diminishes the effect of glutamine in concentrations of the same order of magnitude.

The hypothesis that glutamine-induced growth may be controlled by a specific enzymatic reaction is not discordant with the data presented in Table 1. On these data a test was made to check whether the growth values found do fit the Michaelis-Menten formula, which describes the rate of an enzyme reaction as a function of the concentration of its substrate

$$v = \frac{Vc}{c+K_m}, \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

where v = reaction rate,
c = concentration of substrate,
$V = v$ at $c = \infty$,

and

$K_m = c$ at $v = V/2$.

As growth is found in the embryo culture in the control series, i.e. when glutamine is excluded from the medium, a displacement of the ordinate was assumed, whereby for embryo growth the formula becomes

$$y = \frac{Vc'+Vd}{c'+K_m+d}, \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

where y = growth value in μ at x (= mean of initial length in 48 hr),
c' = concentration of glutamine in mg/l,
V = growth value at $c' = \infty$ in 48 hr,
$K_m = c'$ at $y = V/2$,

and

d = displacement of ordinate in mg/l.

For simplicity the function tested was

$$y = \frac{Vc'+e}{c'+f}. \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (4)$$

Estimation of the parameters of this function, with the six entries of Table 1 fitting by the least squares method, yielded

$$V = 884{\cdot}1\ \mu,$$
$$e = 44116{\cdot}59,$$

and

$$f = 131{\cdot}4.$$

According to a χ^2 test the fit of the entries to equation (4) is quite satisfactory ($\chi^2 = 4{\cdot}8888$, d.f. $= 3$, $0{\cdot}25 > P > 0{\cdot}10$). Thus for equation (3) it follows:

$$V = 884{\cdot}1\ \mu \text{ in 48 hr},$$
$$K_m = 81{\cdot}5 \text{ mg/l } (= 0{\cdot}56 \text{ mM}),$$

and

$$d = 49{\cdot}9 \text{ mg/l } (= 0{\cdot}34 \text{ mM}).$$

The interpretation of the displacement *d* may only be guessed but it surely indicates the extent to which stored nitrogen is made available for residual growth.

The justification of the above mathematical treatment is weakened by two arguments: first, measurement of growth in length may perhaps not be accepted as a true representation of living matter or protein; and second, as the regression coefficients of the growth values do not follow a regular pattern (compare Table 1) the application of the formula may be restricted to the mean initial length of the embryos in this particular experiment.

The tentative explanation of asparagine-induced inhibition has now been complicated by the finding that asparagine did not prove inhibitory through all ranges of concentrations. Low concentrations of 10 mg/l proved slightly growth-promoting, and therefore it must be concluded that some enzyme system for the utilization of the asparagine-N must be present.

Higher concentrations of asparagine inhibit in the absence of glutamine, i.e. when compared with a control which does not contain nitrogen. Previously it was assumed that stored nitrogen for residual growth was made available via glutamine and that external asparagine reduced this residual growth. This may still hold if the asparagine enzyme system contributes so little in the synthetic direction that a large surplus of external asparagine blocks the main glutamine pathway thus effecting an overall growth depression. This is in line with the observation that the growth promotion with relatively low asparagine concentrations is very small indeed.

V. Acknowledgments

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VI. References

Chibnall, A. C. (1939).—"Protein Metabolism of Plants." 1st Ed. (Yale Univ. Press: New Haven.)

Haagen Smit, A. J., Siu, R., and Wilson, G. (1945).—*Science* **101**: 234.

Linderstrøm Lang, K., and Holter, H. (1941).—Die enzymatische Histochemie. In "Die Methoden der Ferment forschung." Bd. 1. p. 1132. (Ed. E. Bamann and K. Myrbäck.) (J. Springer: Berlin.)

Meiss, A. N. (1952).—Bull. Conn. Agric. Exp. Sta. No. 553.

Paris, D., Rietsema, J., Satina, S., and Blakeslee, A. F. (1953).—*Proc. Nat. Acad. Sci., Wash.* **39**: 1205.

Rappaport, J. (1954).—*Bot. Rev.* **20**: 201.

Rietsema, J., Satina, S., and Blakeslee, A. F. (1953).—*Amer. J. Bot.* **40**: 538.

Rijven, A. H. G. C. (1952).—*Acta Bot. Neerl.* **1**: 159.

Rijven, A. H. G. C. (1955).—*Proc. Acad. Sci. Amst.* (series C) **50**: 368.

Steward, F. C., and Thompson, J. F. (1954).—Proteins and protein metabolism in plants. In "The Proteins." Vol. 2. Pt. A. Ch. 19. p. 513. (Ed. H. Neurath and K. Bailey.) (Academic Press Inc.: New York.)

INHIBITION OF TRANSPORT OF INDOLE-3-ACETIC ACID IN THE ETIOLATED HYPOCOTYL OF *PHASEOLUS VULGARIS* L.

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Summary

The effect of a number of auxins and auxin analogues on the basipetal transport of indole-3-acetic acid (IAA) in 5-mm segments of hypocotyls of 5-day-old etiolated *Phaseolus vulgaris* L. seedlings has been investigated.

Donor and receptor agar plates were applied respectively to the morphologically apical and basal cut ends of the segments and the receptor plates assayed for their content of auxin by means of the *Avena* curvature test.

Pre-treatment of the hypocotyl segments with either 2,4-dichlorophenoxyacetic acid, 2,4-dichlorophenoxypropionic acid, 2,4-dichlorophenoxybutryic acid, 2,4,6-trichlorophenoxyacetic acid, indole-3-propionic acid, or 2,3,5-tri-iodobenzoic acid decreased the curvature given by blocks cut from the receptor plates. Pretreatment with either *p*-chlorophenoxy*iso*butyric acid, 2,4-dichloroanisole, coumarin, *o*-*iso*propyl-*N*-phenylcarbamate, indole, indole-3-acetonitrile, or phenylacetic acid was without effect.

Substances inhibiting transport of IAA also decreased the IAA-induced curvature when incorporated with IAA in agar blocks and tested directly in the *Avena* test. These substances, however, were not transported in the *Phaseolus* segments under the conditions used.

It is concluded that these inhibitors affect the mechanism of the basipetal transport of IAA, and as a tentative explanation it is suggested that this is achieved by competition for sites on hypothetical carriers of IAA.

I. Introduction

Since the discovery of auxins it has been recognized that their transport is polar and requires an energy source. Thus rate of transport can be reduced by inhibitors of respiratory metabolism (du Buy and Olson 1940). As a working hypothesis for this study it has been postulated that, in absorption and transport, auxin becomes attached to a carrier in a specific way. If such carrier sites were occupied by structurally-related compounds another type of inhibition would be obtained.

The literature contains a few reports of inhibition of transport of indole-3-acetic acid (IAA) by auxin analogues. Skoog (1954, 1955*a*, 1955*b*) states that tri-iodobenzoic acid (TIBA) has an inhibitory effect on the transport of IAA. In a short report by Niedergang-Kamien (1955) this property has also been attributed to phenylacetic acid.

This paper deals with experiments planned on the working hypothesis mentioned above. During testing of some auxins and auxin analogues, several of them have been found to inhibit transport of IAA. Whilst this finding alone does not provide sufficient evidence for verification of the hypothesis concerning the transport mechanism, it is thought to add to the understanding of the action of a number of synthetic growth substances.

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During the preparation of this manuscript a fuller account of the work on TIBA by Niedergang-Kamien and Skoog (1956) has come to hand. As will become evident (see Section IV) the results mentioned in the present paper provide an independent confirmation of some of their findings.

II. MATERIALS AND METHODS

(a) *Pre-treatment Experiments*

The main conclusions of this work were drawn from the experiments in which hypocotyl segments were pre-treated with auxins and auxin analogues. For these, seeds of *Phaseolus vulgaris* L. var. Brown Beauty from the 1955 harvest were soaked for 2 hr and then germinated and grown for 5 days at 25°C and 85 per cent. R.H. under weak red light. The hypocotyls were then 10–20 cm long. A uniform batch was selected and, from each, two 5-mm segments were cut from the region just beneath the cotyledonary hook with a double-bladed cutting tool. With the segment still held in the cutter, "Vaseline" was smeared around its circumference midway between the ends, using a hypodermic syringe with a No. 18 needle. This minimized the risk of formation of a water film over the segment's surface. After moistening its morphologically basal end, the segment was placed with this end downwards on an 11 by 11 by 1·5 mm 1·5 per cent. agar plate resting on a microscope slide in a Petri dish lined with moist filter paper. Three other identically prepared segments were placed on the same "receptor" plate. Two segments from the higher region of a hypocotyl were placed on each receptor plate, and were distributed so that all such segments had an equal chance of being allocated to any given plate. The same conditions applied with the lower segments.

Each auxin or auxin analogue was incorporated into an agar plate otherwise identical with a receptor plate. Such "pre-treatment" plates (adhering each to a cover-slip) were placed over the moistened apical ends of the segments. The control pre-treatment plate did not contain growth substance. After 1 hr, these plates were removed without disturbing the segments and were replaced with others identical with them, except that they contained IAA instead of the auxin or auxin analogue. This "donor" plate remained in position for 3 hr; it was then removed, together with the hypocotyl segments, and the IAA collected in the receptor was assayed using the *Avena* curvature test.

(b) *The* Avena *Curvature Test*

Victory oats from Svalöf (1949 harvest) were grown at 25°C and 85 per cent. R.H. for 72 hr, using the modification developed by Dr. L. A. T. Ballard of this Division. They were then transferred to modified holders and treated as in the standard test. The 1·5 per cent. agar plates to be tested were cut into blocks 10 mm^3 and applied to the plants held in racks, each of which could hold 12 plants. The number of plants placed in each rack was the same as the number of treatments or a multiple of this number so that each treatment was represented by at least one plant in each rack. Within each rack the treatments were arranged in random order. The advantage of this procedure is that here each plant may be used as a replicate; in the conventional arrangement the mean of 12 plants is a replicate. Unless otherwise

Table 1

EFFECT ON BASIPETAL AUXIN TRANSPORT OF 1-HR PRE-TREATMENT OF PHASEOLUS SEGMENTS WITH AUXINS AND AUXIN ANALOGUES

Means of measured *Avena* curvatures (degrees), means of pooled curvatures, and means of pooled transformed curvatures together with difference for significance given by receptor plates after pre-treatment of *Phaseolus* segments with control (plain agar) and 11 auxins and auxin analogues. F for treatments (11 and 264 d.f.) = 14·7, $P < 0{\cdot}001$. Difference for significance between transformed values at the 1 per cent. level = 0·055

	Control	PARA	DCA	Coumarin	Indole	IAN	2,4-D	2,4-DP	2,4-DB	2,4,6-T	TIBA	IPA
Mean curvatures (degrees) (expt. 1)	6·1	6·6	6·2	5·9	7·0	5·1	2·1	3·2	1·6	2·3	2·2	2·3
Mean curvatures (degrees) (expt. 2)	5·7	8·1	8·2	8·2	6·6	7·1	1·6	2·3	2·5	3·1	2·7	2·1
Pooled means	5·9	7·4	7·2	7·1	6·8	6·1	1·9	2·7	2·0	2·7	2·4	2·2
Pooled transformed means	1·208	1·233	1·220	1·232	1·213	1·196	1·066	1·103	1·073	1·102	1·087	1·087
Significant difference from control	—	—	—	—	—	—	**	**	**	**	**	**

** $P = 0{\cdot}01$.

indicated in the text, 12 racks were used in each experiment: thus there were 12 replicate determinations of the auxin concentration of each plate.

The interval between decapitations was 3 hr and the blocks remained in contact with the coleoptiles for $1\frac{1}{2}$ hr before photographing.

The curvatures were measured and the results treated statistically. On testing it was found that the means and variances of the treatments were not independent. A logarithmic transformation of the measured curvatures was made so that this condition of independence was fulfilled. The analysis of variance was carried out on these transformed figures; thus differences between treatments are indicated by comparison of the means of transformed curvatures.

The synthetic growth substances used were *p*-chlorophenoxy*iso*butyric acid (PARA), 2,4-dichloroanisole (DCA), coumarin, indole, *o*-*iso*propyl-*N*-phenylcarbamate (ISO), 2,4,6-trichlorophenoxyacetic acid (2,4,6-T), 2,4-dichlorophenoxyacetic acid (2,4-D), α-(2,4-dichlorophenoxy)-*n*-butyric acid (2,4-DB), α-(2,4-dichlorophenoxy)-propionic acid (2,4-DP), 2,3,5-tri-iodobenzoic acid (TIBA), indole propionic acid (IPA), indole-3-acetonitrile (IAN), and phenylacetic acid (PAA).

(*c*) *Special Experiments with IAA*

In some experiments IAA was added directly to the receptor plate and after removal of the pre-treatment plate no donor plate was applied. However, the segments remained in position for a further 3 hr; they were then removed and the receptor plate was assayed as in the pre-treatment experiments.

III. RESULTS

The results reported are derived from experiments of three types. The main conclusions come from experiments in which the apical ends of hypocotyl segments were pre-treated with auxins and auxin analogues. These conclusions are extended and supported by experiments in which auxins and auxin analogues were incorporated with IAA into the *Avena* test block and by those in which movement of pre-treatment substance into the receptor plate was investigated.

(*a*) *The Effect of Pre-treatment of Apical Ends of Hypocotyl Segments with Auxins and Auxin Analogues*

The pooled results of two identical experiments in which 11 substances were compared with control are shown in Table 1. The concentration of IAA used throughout was $6 \cdot 0 \times 10^{-6}$M, TIBA was at $1 \cdot 2 \times 10^{-4}$M, and the remaining substances at $2 \cdot 5 \times 10^{-4}$M. On each occasion all 12 *Avena* test blocks were cut from the same receptor plate, but, as the experiment was carried out twice, an estimate of the variance due to differences between receptor plates receiving the same treatment could be made. The analysis of variance showed that this variance was not significant. The results show that pre-treatment with 2,4-D, 2,4-DP, 2,4-DB, 2,4,6-T, TIBA, and IPA reduce curvature markedly compared with the control (plain agar pre-treatment) but that pre-treatment with PARA, DCA, coumarin, indole, or IAN has no significant effect.*

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* In another experiment it was found that PAA pre-treatment had no significant effect.

TABLE 2

EFFECT OF AUXINS AND AUXIN ANALOGUES ON IAA-INDUCED CURVATURE IN THE AVENA TEST

Means of measured *Avena* curvatures in degrees and means of transformed curvatures together with significant differences from five experiments. In the first two experiments the curvatures given by control (plain agar) are compared with those from nine substances alone, and the curvature given by $4{\cdot}5\times10^{-7}$M IAA is compared with those given by each of the nine substances in combination with this concentration of IAA. In the remaining three experiments the indicated comparisons are made using three substances both alone and in combination with $3{\cdot}0\times10^{-7}$M IAA

Expt. No.	Auxin or Auxin Analogue	Concn. (M)	Alone			With IAA $4{\cdot}5\times10^{-7}$M			D.F.	*F*	Differences for Significance between Transformed Values
			Mean Curvatures (degrees)	Transformed Means	Significant Difference from Control	Mean Curvatures (degrees)	Transformed Means	Significant Difference from IAA Alone			
1	Control		1·1	1·044	—				11 and 110	14·7***	0·088*
	IAA	$4{\cdot}5\times10^{-7}$	13·9	1·393	**						0·117**
	2,4-DP	$2{\cdot}5\times10^{-4}$	4·6	1·155	*	3·6	1·137	**			
	2,4-DB	$2{\cdot}5\times10^{-4}$	4·1	1·151	*	2·9	1·114	**			
	2,4,6-T	$2{\cdot}5\times10^{-4}$	3·3	1·126	—	3·3	1·102	**			
	IPA	$2{\cdot}5\times10^{-4}$	4·0	1·149	*	5·1	1·188	**			
	PAA	$2{\cdot}5\times10^{-4}$	3·6	1·139	*	6·4	1·218	**			
2	Control		1·6	1·059					11 and 110	50·6***	0·072*
	IAA	$4{\cdot}5\times10^{-7}$	16·7	1·447	**						0·094**
	DCA	$2{\cdot}5\times10^{-4}$	1·3	1·051	—	15·8	1·428	—			
	Coumarin	$2{\cdot}5\times10^{-4}$	1·7	1·069	—	17·7	1·457	—			
	Indole	$2{\cdot}5\times10^{-4}$	1·7	1·062	—	18·0	1·467	—			
	PARA	$2{\cdot}5\times10^{-4}$	1·3	1·046	—	10·1	1·316	**			
	TIBA	$6{\cdot}0\times10^{-5}$	3·1	1·122	—	4·7	1·175	**			

* $P = 0{\cdot}05$. ** $P = 0{\cdot}01$. *** $P < 0{\cdot}001$.

TABLE 2 (*Continued*)

Expt. No.	Auxin or Auxin Analogue	Concn. (M)	Alone			With IAA $3{\cdot}0\times10^{-7}$M			D.F.	*F*	Differences for Significance between Transformed Values
			Mean Curvatures (degrees)	Transformed Means	Significant Difference from Control	Mean Curvatures (degrees)	Transformed Means	Significant Difference from IAA Alone			
3	IAA	$3{\cdot}0\times10^{-7}$	11·4	1·319					—	—	0·089*
	2,4-D	$2{\cdot}5\times10^{-6}$	1·8	1·057		4·8	1·157	**			0·118**
	2,4-D	$2{\cdot}5\times10^{-7}$				6·6	1·210	*			
4	Control		0·9	0·994	—				3 and 33	62·3***	0·069*
	IAA	$3{\cdot}0\times10^{-7}$	12·3	1·335	**						0·093**
	ISO	$2{\cdot}5\times10^{-4}$	1·6	1·059	—	14·4	1·337	—			
5	IAA	$3{\cdot}0\times10^{-7}$	12·9	1·345					4 and 223	17·7***	0·025*
	IAN	$3{\cdot}0\times10^{-7}$	11·6	1·307		16·5	1·418	**			0·039**
	IAN	$6{\cdot}0\times10^{-7}$	12·6	1·336		14·8	1·389	**			

* $P = 0{\cdot}05$. ** $P = 0{\cdot}01$. *** $P < 0{\cdot}001$.

An obvious explanation of this reduction in curvature after certain of the pre-treatments would be that in these cases the movement of IAA through the *Phaseolus* segments has been inhibited. However, if any of these auxins or auxin analogues were to give *Avena* curvatures themselves or to interact with IAA in the *Avena* test, the results might be explained by their transport to the receptors during the pre-treatment period. Accordingly we have carried out one series of experiments to determine whether any of these substances have curvature activity or influence IAA-induced curvature, and another to test for their transport under the pre-treatment conditions.

(*b*) *The Effect of Adding Certain Auxins and Auxin Analogues to IAA in the* Avena *Test*

The results of five experiments in which these substances were tested alone and in combination with IAA in the *Avena* test are shown in Table 2. In two experiments (1 and 2) the IAA concentration was $4 \cdot 5 \times 10^{-7}$M; in the remaining three it was $3 \cdot 0 \times 10^{-7}$M. All the substances except TIBA, 2,4-D, and IAN were at a concentration of $2 \cdot 5 \times 10^{-4}$M, TIBA was at $6 \cdot 0 \times 10^{-5}$M, IAN at $3 \cdot 0 \times 10^{-7}$M, and 2,4-D at $2 \cdot 5 \times 10^{-6}$M and $2 \cdot 5 \times 10^{-7}$M. In the first two experiments the means are obtained from 11 replicate determinations all taken from the same plate; in the third and fourth from 12 replicates from the same plate; and in the fifth from 12 replicates from each of four plates, i.e. 48 altogether.

It is clear from Table 2 that some of the substances give slight curvature in the *Avena* test. However, it is also evident that IAA-induced curvature is markedly diminished when certain of them are incorporated with IAA in the test block. Those having this property are IPA, 2,4-D, 2,4-DP, 2,4-DB, 2,4,6-T, TIBA, PAA, and PARA. The entries show that 2,4-D is still effective in reducing curvature at concentrations as low as $2 \cdot 5 \times 10^{-7}$M. The result for PARA indicates that although it does inhibit IAA-induced curvature it is much less effective than TIBA, the other inhibitor used in this experiment. On the other hand, indole, coumarin, DCA, and ISO do not reduce IAA-induced curvature.

In the case of IAN lower concentrations were used, viz. $3 \cdot 0 \times 10^{-7}$M and $6 \cdot 0 \times 10^{-7}$M, both alone and in combination with $3 \cdot 0 \times 10^{-7}$M IAA. It is seen that IAN alone gives considerable curvature, though less than equimolar IAA. When combined with IAA the curvature is greater than that given by either substance alone, but the combination with IAN concentration of $3 \cdot 0 \times 10^{-7}$M gives a greater curvature than that with IAN concentration of $6 \cdot 0 \times 10^{-7}$M. These results are in general agreement with the observations of Bentley and Bickle (1952).

Hence it has been shown that some of the auxins and auxin analogues reduce IAA-induced curvature in the *Avena* test. Thus, unless it is demonstrated that these substances do not penetrate to the receptor plates during pre-treatment it cannot be concluded that transport in the *Phaseolus* segments has been inhibited. The following experiments were undertaken to discover whether or not such penetration took place.

(*c*) *Transport of Auxins and Auxin Analogues other than IAA*

In these experiments the results are affected by the fact that contact with the cut surfaces of hypocotyl segments reduces the IAA content of receptor plates. This is shown in Table 3 which illustrates the increase of this effect with time of contact. In one experiment subsequently described in which there was a 3-hr contact between segments and receptor plates the initial concentration of IAA was increased to compensate for this effect.

TABLE 3

EFFECT OF PHASEOLUS SEGMENTS ON AUXIN CONTENT OF RECEPTOR PLATES

Means of measured *Avena* curvatures in degrees and means of transformed curvatures together with differences for significance given by IAA $3 \cdot 0 \times 10^{-7}$M and by the same concentration after contact with *Phaseolus* segments

Time of Contact (hr)	Mean Curvatures (degrees)	Transformed Means	Significant Difference from No Contact	D.F.	*F*	Differences for Significance between Treatment Means
0	11·4	1·325	—	2 and 22	11·7***	0·077*
1½	7·7	1·240	*			0·104**
3	4·4	1·143	**			

* $P = 0 \cdot 05$. ** $P = 0 \cdot 01$. *** $P < 0 \cdot 001$.

In two of these experiments (Table 4) an agar plate containing $3 \cdot 0 \times 10^{-7}$M IAA was the receptor whilst the pre-treatment plate contained auxin or auxin analogue at $2 \cdot 5 \times 10^{-4}$M, in all cases, except that of TIBA which was at $1 \cdot 2 \times 10^{-4}$M. The pre-treatment plate remained in position for 1 hr and was then removed; the segments were left for a further 3 hr and after their removal the receptor was assayed by the *Avena* test. In the third experiment (Table 5) IAA was at $6 \cdot 0 \times 10^{-7}$M and the pre-treatment plates remained in position for 3 hr before removal together with the segments.

Table 4 shows the results of two experiments in which each treatment mean is obtained from 12 replicate blocks from the same plate. In the first, 10 auxins and auxin analogues have been compared with the control (plain agar pre-treatment plate). If any of the inhibitors of IAA-induced curvature were reaching the receptors we should expect the curvature in these cases to be less than for control or for the non-inhibitor pre-treatment. The variance ratio test indicates that the observed deviations of the treatment means from the general mean would occur by chance more than once in five. Similarly with the three substances of the second experiment the deviations of the treatment means from the general mean could occur by chance more than once in five. Hence in both cases there is no reason to believe that the inhibitors are affecting the curvature.

Each treatment mean of Table 5 is derived from 12 replicate blocks from each of four plates, i.e. 48 altogether. Under the conditions of this experiment the inhibitors would be even more likely to appear in the receptor plates. The results show no evidence of inhibition of curvature and the variance ratio test indicates that they would be obtained by chance more than once in five. Hence again there is no reason to believe that the inhibitors are affecting the curvature.

Table 4

EFFECT OF 1-HR PRE-TREATMENT OF PHASEOLUS SEGMENTS WITH AUXINS AND AUXIN ANALOGUES ON CURVATURE GIVEN BY IAA INCORPORATED IN THE RECEPTOR PLATES

Means of measured *Avena* curvatures in degrees and means of transformed curvatures given by control (plain agar pre-treatment) and pre-treatment with 13 auxins and auxin analogues in two experiments designed to test for transport of auxins and auxin analogues

Expt. No.	Auxin or Auxin Analogue	Mean Curvatures (degrees)	Transformed Means	D.F.	*F*
1	Control	5·8	1·190	11 and 110	<1*
	PARA	5·2	1·178		
	DCA	4·8	1·166		
	Coumarin	5·7	1·178		
	Indole	6·7	1·216		
	IAN	6·3	1·187		
	2,4-D	5·1	1·175		
	2,4-DP	6·4	1·189		
	2,4-DB	6·1	1·197		
	2,4,6-T	4·1	1·143		
	IPA	3·9	1·137		
2	Control	4·4	1·143	3 and 33	1·2*
	ISO	5·7	1·193		
	TIBA	3·9	1·137		
	PAA	4·1	1·137		

* *P*>0·2.

Table 5

EFFECT OF 3-HR PRE-TREATMENT OF PHASEOLUS SEGMENTS WITH AUXINS AND AUXIN ANALOGUES ON CURVATURE GIVEN BY IAA INCORPORATED IN THE RECEPTOR PLATES

Means of measured *Avena* curvatures in degrees and means of transformed curvatures given by control (plain agar) and pre-treatment with two substances in an experiment designed to test for transport of auxins and auxin analogues

Auxin or Auxin Analogue	Mean Curvatures (degrees)	Transformed Means	D.F.	*F*
Control	12·2	1·334	2 and 108	2·2*
2,4-D	13·4	1·362		
TIBA	12·0	1·332		

* *P*>0·2.

IV. Discussion

Several of the auxins and auxin analogues used in the experiments involving pre-treatment of apical ends of hypocotyl segments cause a reduction in receptor-induced curvature compared with the control pre-treatment. Those which exhibit this property are 2,4-D, 2,4,DP, 2,4-DB, 2,4,6-T, TIBA, and IPA, whilst PARA, DCA, coumarin, ISO, indole, IAN, and PAA have no activity.

Although several explanations could account for this effect, our experiments strongly suggest that it is due to a direct inhibition of transport in the *Phaseolus* segments. It has been shown that the reduced curvature after pre-treatment of *Phaseolus* segments with some of the auxins and auxin analogues cannot be accounted for by the demonstrated inhibition of IAA-induced curvature in the *Avena* test by the same substances. Hence it seems likely that the action of these substances is on the *Phaseolus* segments. These are able to destroy IAA, probably by IAA oxidase activity. Goldacre, Galston, and Weintraub (1953) have shown that 2,4-dichlorophenol enhances the activity of IAA oxidase. If all of the inhibitors contained 2,4-dichlorophenol as an impurity our pre-treatment and *Avena* test results might be explained by enhanced IAA destruction. However, such an explanation is not probable, for whilst 2,4-dichlorophenol might be expected as a contaminant of 2,4-D, 2,4-DP, and 2,4-DB it would not be expected in IPA. On the other hand, DCA and PARA which are inactive might be expected to contain amounts comparable to 2,4-D, 2,4-DP, and 2,4-DB.

Thus it is concluded that the inhibitors are affecting the IAA transport mechanism. As indicated in the Introduction, two types of inhibition may be visualized. Substances such as potassium cyanide and 2,4-dinitrophenol inhibit auxin transport by their effect on energy-transfer reactions. On the other hand, all our inhibitors are structurally related to growth substances.

It is noticeable that with the exception of PARA and PAA all the substances which inhibit IAA-induced curvature in the *Avena* test also inhibit transport in the pre-treatment experiments. This appears less remarkable when it is remembered that auxin transport in the coleoptile is involved in the *Avena* curvature test. Indeed we believe that the two effects arise from the same cause, namely the inhibition of auxin transport. A probable explanation of the exceptions is that the *Avena* test interaction is a more sensitive test for transport inhibition than the *Phaseolus* test, and that PAA and PARA are weak inhibitors of transport. Niedergang-Kamien (1955) lists PAA as an inhibitor of auxin transport.

All substances found active in transport inhibition contain a carboxyl group. The importance of this group is illustrated by the contrasting effects of 2,4-D (active) and 2,4-dichloranisole (inactive). However, the mere possession of a carboxyl group does not imply activity. Further experiments, not reported here, show that neither valeric nor benzoic acid reduces IAA-induced curvature of *Avena* coleoptiles and thus exhibit no evidence of inhibition of auxin transport.

It is therefore suggested that the action of the inhibitors is to compete with IAA for specific "carrier sites". This hypothesis may seem difficult to reconcile with the fact that the inhibitors are not themselves transported. However, even after 3 hr no evidence of their transport was obtained.

Niedergang-Kamien and Skoog (1956) have recently published an account of strikingly similar experiments with sunflower stem cylinders. They demonstrate a decrease in basipetal IAA transport in such sections after a preceding immersion in solutions of TIBA. They relate this result to the morphogenetic effects of TIBA, especially to its effect on the distribution pattern of callus-like outgrowths from tobacco stem segments cultured on a nutrient medium. In TIBA-treated segments these outgrowths occur evenly over the segment surface, in controls they are found only at the basal end. Also the distribution of ether-extractable auxin remains approximately even over the length of the segment after TIBA treatment; in controls the basal concentration becomes higher than the apical.

One criticism which might be made of the work of these authors is the failure to demonstrate that the lowered *Avena* curvatures of the transport experiments were not due to a direct effect of TIBA on IAA-induced curvature in the *Avena* test. Indeed the risk of such an effect would appear to be greater from their immersion pre-treatment than from our agar plate method.

Niedergang-Kamien and Skoog (1956) have suggested that the morphogenetic effects of TIBA can be explained by its effect on basipetal auxin transport alone. However, in the same paper, they also state that other substances have a similar effect on auxin transport. This statement coupled with the results of this paper raises the questions whether other substances give the same morphogenetic effects as TIBA, and if not whether Niedergang-Kamien and Skoog's suggested explanation is sufficient.

The physiological activity of herbicides such as 2,4-D is not well understood. Although many effects of 2,4-D on plants have been demonstrated, this is, in our belief, the first account of its inhibition of basipetal auxin transport. This may be an important part of its herbicidal action.

V. Acknowledgments

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VI. References

Bentley, J. A., and Bickle, A. S. (1952).—*J. Exp. Bot.* **3**: 406.

du Buy, H. G., and Olson, R. A. (1940).—*Amer. J. Bot.* **27**: 401.

Goldacre, P. L., Galston, A. W., and Weintraub, R. L. (1953).—*Arch. Biochem. Biophys.* **43**: 358.

Niedergang-Kamien, E. (1955).—*Plant Physiol.* **30** (suppl.): v.

Niedergang-Kamien, E., and Skoog, F. (1956).—*Physiol. Plant.* **9**: 60.

Skoog, F. (1954*a*).—Abnormal and pathological plant growth. In "Brookhaven Symposia in Biology." No. 6. p. 1. (Brookhaven Natl. Lab.: New York.)

Skoog, F. (1954*b*).—In "Dynamics of Growth Processes." Ch. 8. p. 175. (Ed. E. J. Boell.) (Princeton Univ. Press: New Jersey.)

Skoog, F. (1955).—*Ann. Biol.* **31**: 201.

THE EFFECT OF MINERAL NUTRITION ON THE CONTENT OF FREE AMINO ACIDS AND AMIDES IN TOMATO PLANTS

I. A COMPARISON OF THE EFFECTS OF DEFICIENCIES OF COPPER, ZINC, MANGANESE, IRON, AND MOLYBDENUM

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Summary

The level and the quantitative composition of the free amino acid fraction of tomato plants grown in full nutrient and in cultures deficient in zinc, copper, manganese, iron, and molybdenum have been determined. The methods used in the investigation include the quantitative estimation of amino acids by a technique involving paper chromatography, and the culture of tomato plants in highly purified nutrient solutions.

All the deficiencies investigated except that of molybdenum brought about increases in the free amino acid fraction. In every case there was a change in the quantitative composition of this fraction. Iron and zinc deficiencies were associated with large increases in the free levels of the two amides asparagine and glutamine, while with copper and manganese deficiencies many individual amino acids increased in concentration but not the two amides. The level of total free amino acids and amides was low in molybdenum-deficient plants.

The qualitative differences between deficient- and full-culture plants were not large, but included the accumulation of β-alanine with deficiency of zinc, copper, or molybdenum, and of pipecolinic acid with deficiency of manganese or iron. Other differences were the absence of histidine with copper, manganese, iron, and molybdenum deficiency, of phenylalanine with copper deficiency, and of lysine with copper, manganese, and molybdenum deficiencies. Additionally many quantitative changes in amino acid concentration were found. In the deficient plants there were changes in the concentration of most amino acids relative to the levels in the controls. These changes were not uniform and the relative amounts of the different amino acids were altered with each deficiency.

The significance of these results is discussed in relation to the known interactions between the mineral nutrition and the amino acid composition of plants. Although a characteristic spectrum of free amino acids is associated with each individual deficiency, the differences are not considered sufficiently large to make analysis for these compounds an additional method for the diagnosis of mineral deficiencies in this plant.

I. Introduction

The importance of amino acids in the metabolism of plants has occasioned a number of investigations on the relation between these compounds and mineral nutrition. Richards and Templeman (1936) and Gregory and Sen (1937) were among the first to show an effect of the mineral nutrient supply on the concentrations of free amino acids and of amides. These workers found enhanced concentrations in phosphorus- and potassium-deficient, and depressed concentrations in nitrogen-deficient, barley plants. Subsequently accumulations of free amino nitrogen

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compounds were demonstrated by Eaton (1941) in sulphur-deficient sunflower plants, by Bean (1942) in zinc-deficient tomato plants, by Steinberg, Bowling, and McMurtry (1950) in calcium-, magnesium-, and boron-deficient tobacco plants, and by Hewitt, Jones, and Williams (1949) in manganese-deficient cauliflowers. Hewitt *et al.*, however, also showed that molybdenum deficiency is accompanied, not by a high, but by an abnormally low content of free amino nitrogen compounds.

Recently the effect of mineral nutrition on the composition as well as the concentration of the free amino acid fraction has been investigated by Richards and Coleman (1952) and Richards and Berner (1954), whose most important observation was that of an accumulation of the diamine putrescine in potassium-deficient barley leaves.

Hitherto, however, data have not been available on the relation between micronutrient deficiency and the level, as well as the composition, of the free amino acid fraction. It has been the purpose of this investigation to supply such quantitative data. Tomato was chosen as the test plant and the effects of deficiencies of copper, zinc, iron, manganese, and molybdenum were investigated.

The tomato has been used as the experimental material since it grows well in water culture (Hoagland and Arnon 1938), and the symptoms of deficiencies of all the known essential elements in this species have been described (Hambidge 1941; Wallace 1951). The analytical methods employed have included the technique of paper chromatography, which has enabled quantitative estimations to be made of all the individual free amino acids and amides present in these plants.

The primary significance of results from this, as from all other similar investigations, is that they show the extent to which particular elements are involved in the metabolism of amino acids in general. At the same time earlier results have shown that extensive changes are promoted by mineral deficiencies and the situation therefore holds the possibility that an analysis of the amino acid fraction may provide a basis for the identification of the deficiency. This was a primary consideration in the design of the investigation.

II. METHODS

(*a*) *Culturing of Experimental Plants*

Seeds of tomato (*Lycopersicon esculentum* Mill. var. Pan America) were germinated on waxed mosquito netting over distilled water. After approximately 3 weeks, the seedlings were transplanted into nutrient solutions in 3-l. "Pyrex" beakers fitted with aeration tubes. Eight plants were grown per beaker and the basic composition of the culture solutions was essentially the same as that recommended by Stout and Arnon (1939).

Nutrient mixtures deficient in molybdenum were prepared by the method of Gentry and Sherrington (1950) in which this element is removed from macronutrient solutions by chelation with 8-hydroxyquinoline. It was found that this treatment also removes iron. Copper-, zinc-, and manganese-deficient nutrient mixtures were prepared by the method of Stout and Arnon (1939).

The control plants were grown in culture solutions prepared using the purified nutrient solutions, and adding a full complement of trace elements. The micronutrient solutions were prepared from "Analar" grade salts without any prior purification.

(*b*) *Preparation and Purification of Plant Material Extracts*

Extraction of the free amino acids and amides from the plant material was carried out by grinding in cold 80 per cent. ethanol (Woodward and Rabideau 1953). The grinding was carried out for 10 min using a top drive homogenizer, operating at a speed of 1600 r.p.m. The alcohol suspension was allowed to stand overnight in the cold after which it was centrifuged. The insoluble material was resuspended in 80 per cent. ethanol and after centrifugation the two supernatant fractions were bulked. Approximately 5 g fresh weight of plant material were used to prepare one ethanol extract which had a final volume of 250 ml.

The ethanol extract, although it carried no protein, did contain various other substances which could disturb the chromatographic analysis for amino acids. These were separated by evaporating the extract to dryness at 40°C *in vacuo*, and redissolving the residue in water, which was then poured on to a column of "Zeokarb 225" which had previously been brought to the H^+ form. Sugars and salts were removed by washing the column with distilled water and finally the amino acids were released from the resin by washing with 1·5N ammonium hydroxide. This ammonia extract was evaporated to dryness *in vacuo* and the amino acids and amides were dissolved in a known volume, usually 1·0 ml of a 10 per cent. *iso*propanol–water mixture.

The sulphonated crosslinked polystyrene resin "Zeokarb 225" was used, as tests with standard amino acid solutions showed that all the amino acids and amides commonly occurring in plant material could be quantitatively eluted with 1·5N ammonium hydroxide. The only exception was cysteic acid which was completely lost in tests using standard solutions. This substance was either not held on the column or could not be eluted with ammonia. The closely related and naturally occurring amino acid cystine was not lost with resin column treatment.

(*c*) *Estimation of Amino Acids and Amides*

Qualitative analyses of unpurified plant material extracts were made by two-dimensional paper chromatography using the ascending method of solvent flow (Williams and Kirby 1948). A phenol–water mixture (80:20 w/v) was used as the first solvent, followed by the *n*-butanol–acetic acid–water mixture (4:1:1 v/v) of Reed (1950) in the transverse direction. To confirm the identity of various amino acids, *n*-propanol–water (70:30 v/v) and ethanol–water (15:25 v/v) were used as alternative second solvents. In some cases the phenol solvent was modified by the addition of acetic acid or ammonia vapours (Consden, Gordon, and Martin 1944; Dent 1948).

The general detecting procedure was by spraying with 0·1 per cent. ninhydrin in ethanol followed by heating. An isatin-in-ethanol spray followed by heating was used to detect proline. Modified Sakaguchi and Pauli tests were used to confirm the identity of arginine and histidine respectively (Sakaguchi 1950; Sanger and Tuppy 1951).

For the quantitative analyses two-directional paper chromatography was again employed, but under highly standardized conditions. All the chromatographic separations were carried out at a standard temperature which for ease of maintenance was 27±0·5°C.

Again a phenol–water mixture (80:20 w/v) was the first solvent, and using the ascending method of solvent flow, in an atmosphere of ammonia and cyanide vapours, a sufficient separation was obtained in 30 hr. In this case no prior equilibration of the paper with the atmosphere of the solvent tank was necessary. The second solvent of *n*-butanol–acetic acid–water mixture (4:1:1 v/v) was applied by the descending technique. With this more volatile solvent equilibration of the paper with the atmosphere of the solvent vapours for 4 hr prior to commencing the solvent run of 20 hr was essential for consistent results. Whatman No. 1 papers were used throughout as they gave reproducible results with this two-solvent system. Accurately-calibrated micropipettes were used to transfer the aliquots of between 5 and 50 μl of plant extract to the paper chromatograms. The papers, wet with solvent, were dried in a forced draught of air at 30°C and with this treatment decomposition of the amino acids was negligible (Novellie 1950).

The amino acids and amides separated by paper chromatography were estimated essentially as described by Wellington (1952). Papers were sprayed with a mixture of 2 per cent. ninhydrin and 0·1 per cent. pyridine in ethanol, and the colours were developed for 24 hr under the standard conditions of 40 per cent. relative humidity at a temperature of 20°C. The coloured areas which developed were eluted in 50 per cent. aqueous ethanol and colour intensity measurements were made using a Coleman spectrophotometer. All the measurements were made at 570 mμ, including that of the brown asparagine-ninhydrin complex.

Proline and hydroxyproline, which give yellow colours with ninhydrin, were not estimated with this reagent since they give a more sensitive reaction with isatin (Acher, Fromageot, and Jutisz 1950). When the papers are sprayed with a saturated solution of isatin in ethanol and heated at 65°C for 30 min no amino acids or amides other than proline and hydroxyproline give blue colours. Fortunately none of the plant material extracts, when analysed using a two-solvent system, showed the presence of hydroxyproline and the specificity of the isatin reaction with proline could be used to estimate this constituent directly. The intensity of the blue isatin-proline colour, which cannot be eluted from the paper, was measured directly on the paper by transmittance densitometry using a recording photoelectric densitometer.

The significance of the quantitative results obtained by the procedure outlined above was examined by preparing calibration curves with pure amino acids. A series of chromatograms was prepared using serial dilutions of standard amino acid solutions and the intensities of the blue amino acid-ninhydrin colours were measured spectrophotometrically. Individual calibration curves were obtained for each amino acid by plotting the mean values of six replicate estimations of percent. transmission at a series of six different concentrations.

With ninhydrin as the colour reagent the relationship of amino acid concentration to the logarithm of percent. transmission was a straight line but the slope of this line varied for each individual amino acid. Straight lines were fitted by the method of least squares to the logarithms of percent. transmission against amount of amino acid in micrograms. The line passed in each case through the reading for the blank, which was based on a large number of observations, with high reproducibility. A test was made for excessive deviation from the line of the mean log transmissions at the concentrations used relative to the pooled replicate variance about the means. The deviations were significant only in the case of asparagine and leucine and there was no obvious curvilinear trend in any instance with the possible exception of leucine.

TABLE 1

RANGE OF RELATIVE PRECISION OF THE PAPER CHROMATOGRAPHIC METHOD FOR THE QUANTITATIVE DETERMINATION OF AMINO ACIDS

Amino Acid or Amide	Range of Concentration over which Relative Precision Estimated (μg)	Relative Precision of an Individual Estimation (μg)
Aspartic acid	0–19·94	0·920
Glutamic acid	0–22·08	1·118
Asparagine	0–19·80	1·198
Glutamine	0–21·90	0·679
Citrulline	0–26·28	0·771
Histidine	0–23·28	1·180
Lysine	0–21·94	1·303
Arginine	0–26·12	1·300
Serine	0–15·78	0·625
Glycine	0–11·28	0·424
Threonine	0–17·88	0·567
α-Alanine	0–13·38	0·238
β-Alanine	0–13·38	0·690
γ-Aminobutyric acid	0–15·48	0·487
Ethanolamine	0– 9·16	0·423
Pipecolinic acid	0–19·34	0·922
Phenylalanine	0–24·78	1·455
Valine	0–17·58	0·572
Leucine	0–19·68	0·847
Proline	At concn. of 4·0 μg	0·690

The mean square for deviates of individual logarithms of percent. transmission from the line, and their corresponding standard error were computed. This standard error divided by the slope of the line is an approximate estimate of the error of estimation of amino acid concentration corresponding to a particular logarithm of percent. transmission. A summary of this approximate precision with which each amino acid was estimated when using ninhydrin is given in Table 1.

Proline was considered separately as the relationship between the amount of proline and the logarithm of percent. transmission, as measured by the densitometer, was not a straight line. A smooth curve was fitted in this case and provided the calibration curve. An assessment of the accuracy of an individual proline estimation at the set concentration of 4·0 μg was made, as many of the actual proline estimations were carried out in the range between 3 and 5 μg by carrying out serial dilutions of the extracts.

Although the individual calibration lines were all drawn through the reading for the blank, which corresponds to an amino acid concentration of zero, the smallest amount of each amino acid that could be detected was determined by the approximate precision with which each could be estimated. These values are presented in Table 1 and vary between 0·238 μg for α-alanine and 1·455 μg for phenylalanine. When analysing each plant material extract a series of chromatograms was prepared using different amounts of the extract. In this way most of the actual estimates of concentration were carried out using the central portions of the calibration lines and all the free amino acids present in the plant material at concentrations of greater than approximately 0·05 μg per mg dry weight were estimated. Accordingly amino acids referred to as absent may have been present at concentrations of less than this value.

(*d*) *Experimental Design*

The present investigation involved two different experimental series. In the first, the effects of molybdenum and iron deficiency were investigated and the plants were harvested 2 weeks after transplanting and 5 weeks after the seed was sown. In the second, the effects of zinc, copper, and manganese deficiency were examined, and the plants were harvested 4 weeks after transplanting and 7 weeks after sowing the seed. Each series involved a separate control culture provided with a complete nutrient mixture. In each series the harvest was delayed until the appropriate deficiency symptoms had been fully developed.

The analyses are based on the whole shoot. The stem was cut immediately above the cotyledons, and separate samples were taken for amino acid and dry weight determinations. The analytical results for molybdenum deficiency are based on five separate samples, those for iron and the corresponding control on single samples, and those for zinc, copper, and manganese deficiency and their corresponding control on two samples each. The samples taken for the determination of dry weight involved from four to six whole shoots taken at random from the plants that were harvested. The samples taken for the estimation of amino acids consisted of 15 shoots in each of the molybdenum-deficient samples, 24 shoots in the iron-deficient sample, and six shoots in the corresponding control sample. Six shoots were involved in each sample of the copper-, zinc-, and manganese-deficient material and six in the sample from their corresponding control.

III. Results

The primary data obtained in this investigation are given in Table 2. However, the significance of the quantitative differences can only be assessed from the logarithmic transformations of these figures which are given in Table 3 with the appropriate significance values. In the statistical analysis a log transformation to equalize variances within treatments for each constituent was made and the separate estimates of variance were pooled. These pooled variances were assumed to be applicable to all treatment comparisons. This assumption may not be strictly valid but the significance levels given should serve as reasonable reference values. In Table 3 the values for the individual constituents of the iron- and molybdenum-deficient plants have been adjusted for the difference between the controls of series 1 and 2. This adjustment facilitates comparison of the iron and molybdenum deficiency values with the common control values and with the zinc, copper, and manganese deficiency values.

TABLE 2

COMPARISON OF THE EFFECTS OF DIFFERENT DEFICIENCIES ON THE CONCENTRATIONS OF FREE AMINO ACIDS AND AMIDES AND ON DRY WEIGHT

Results are expressed as μg of amino acid or amide per mg dry weight

Amino Acid	Control 2*	—Zn	—Cu	—Mn	Control 1**	—Fe	—Mo
Aspartic acid	1·526	4·408	5·553	4·996	0·985	2·303	0·152
Glutamic acid	5·107	7·624	7·116	6·683	5·848	6·911	2·500
Asparagine	0·343	16·58	0·738	0·545	0·415	6·307	0·161
Glutamine	3·850	26·27	2·277	1·400	2·180	10·27	0·306
Citrulline	0·447	Absent	0·399	0·407	0·199	0·582	0·254
Histidine	0·129	1·031	Absent	Absent	0·093	Absent	Absent
Lysine	0·068	0·749	Absent	Absent	0·063	0·340	Absent
Arginine	0·183	1·058	0·350	0·328	0·210	0·503	0·445
"Under arginine"	0·043	0·309	0·182	0·271	0·034	0·275	0·288
Serine	1·770	3·116	1·345	0·482	1·258	1·152	1·460
Glycine	1·810	2·474	0·755	0·202	0·797	0·548	0·191
Threonine	1·063	2·214	0·767	0·898	0·629	0·880	0·238
α-Alanine	1·730	1·823	1·359	1·166	1·299	0·895	0·885
β-Alanine	Absent	0·436	0·443	Absent	Absent	Absent	0·141
γ-Aminobutyric acid	0·439	0·527	0·328	0·532	0·184	0·495	0·924
Ethanolamine	0·288	Absent	0·241	0·330	0·220	1·125	0·226
Phenylalanine	0·194	1·355	Absent	0·192	0·239	0·329	0·178
Pipecolinic acid	Absent	Absent	Absent	0·385	Absent	0·178	Absent
Valine	0·234	1·026	0·295	0·288	0·189	0·453	0·203
Leucine	0·266	1·892	0·298	0·358	0·226	0·604	0·267
Proline	0·681	1·505	2·022	0·766	0·545	1·133	0·843
Total amino acids	15·98	31·57	21·45	18·28	13·01	17·71	9·195
Total amides	4·193	42·85	3·015	1·945	2·595	16·58	0·467
Dry weight (mg per plant)	213·6	65·95	75·77	69·41	79·52	10·18	17·98

* Control 2 comparable only with —Zn, —Cu, —Mn.

** Control 1 comparable only with —Fe and —Mo.

Before considering the changes promoted by the different deficiencies it may be noted that only one substance could not be identified. This substance reacted with ninhydrin but not with isatin, and had the same R_F as arginine with the phenol solvent but occupied a position below it with the butanol–acetic acid–water solvent. It was estimated with the arginine calibration curve and is referred to as "under arginine".

The effects of the deficiencies may be considered by comparing the data for each group with those for the control.

Molybdenum.—The most striking effect here is the low total amino acid content. Other effects include an absence of histidine and lysine which are present in the control and the presence of β-alanine which is absent from the control. Another feature is the very high level of γ-aminobutyric acid in these plants.

Table 3

COMPARISON OF EFFECTS OF DIFFERENT DEFICIENCIES ON THE CONCENTRATIONS OF FREE AMINO ACIDS AND AMIDES

Transformation log (× 10,000) of values for μg of amino acid or amide per mg dry weight

Amino Acid	Control 2	−Zn	−Cu	−Mn	−Fe*	−Mo*	Least Diff. (5%) between Zn, Cu, Mn, and Control	Least Diff. (5%) between Fe, Mo, and Control	Least Diff. (5%) between Fe or Mo and Cu, Zn, Mn
Aspartic acid	3·184	3·644	3·745	3·697	3·553	2·372	0·098	0·139	0·240
Glutamic acid	3·701	3·881	3·851	3·815	3·773	3·332	0·259	0·366	0·634
Asparagine	2·534	4·220	2·868	2·715	3·716	2·123	0·278	0·393	0·681
Glutamine	3·565	4·418	3·357	3·137	4·239	2·713	0·259	0·366	0·634
Citrulline	2·645	Absent	2·602	2·608	3·111	2·751	0·170	0·240	0·416
Histidine	2·109	3·013	Absent	Absent	Absent	Absent	0·114	—	—
Lysine	1·829	2·869	Absent	Absent	2·561	Absent	0·258	0·365	0·632
Arginine	2·263	3·025	2·541	2·485	2·642	2·589	0·344	0·486	0·842
"Under arginine"	1·632	2·490	2·250	2·410	2·542	2·560	0·351	0·496	0·860
Serine	3·247	3·488	3·095	2·684	3·208	3·311	0·378	0·534	0·926
Glycine	3·242	3·393	2·868	2·250	3·080	2·622	0·536	0·758	1·313
Threonine	3·005	3·342	2·883	2·953	3·150	2·583	0·309	0·437	0·757
α-Alanine	3·238	3·261	3·134	3·064	3·076	3·071	0·105	0·148	0·257
β-Alanine	Absent	2·665	2·642	Absent	Absent	2·270	0·158	—	0·387
γ-Aminobutyric acid	2·642	2·722	2·516	2·725	3·072	3·343	0·089	0·126	0·218
Ethanolamine	2·460	Absent	2·382	2·492	2·215	2·472	0·342	0·483	0·838
Phenylalanine	2·274	3·132	Absent	2·285	2·413	2·146	0·243	0·344	0·595
Pipecolinic acid	Absent	Absent	Absent	2·050	2·371	Absent	—	—	—
Valine	2·367	3·010	2·463	2·455	2·747	2·399	0·236	0·334	0·578
Leucine	2·424	3·275	2·465	2·546	2·851	2·497	0·238	0·337	0·583
Proline	2·835	3·156	3·294	2·878	3·153	3·025	0·368	0·520	0·901

* The transformed values of each constituent of the −Fe and −Mo plants were adjusted for the difference between control 1 (see Table 2) and control 2. The three least difference values allow all combinations of comparisons between control, −Zn, −Cu, −Fe, −Mn, and −Mo to be made.

Zinc.—This deficiency, like all the others in the series except molybdenum, is accompanied by a high total amino acid content. It resembles iron deficiency in being characterized by a very high amide content. Increases in the majority of the individual amino acids contribute to the high total level, with the notable exceptions of citrulline and ethanolamine both of which were absent. Additionally, β-alanine is clearly present in this material.

Iron.—With this deficiency there is a large increase in the total amide level with a smaller increase in the total amino acid level. Further differences are the accumulation of pipecolinic acid, a compound not present in the controls, and the absence of histidine.

Copper.—Here there is an increase in the total amino acid level but little change in the total amide level. Histidine, lysine, and phenylalanine are absent. Phenylalnine is present in the controls and in all the other deficiencies but is characteristically absent in copper-deficient plants.

Manganese.—With this deficiency, as with those of copper and molybdenum, the basic amino acids histidine and lysine are not present in the free pool of amino acids. There is a general increase in the total level of free amino acids, but again no change in the amide fraction. The compound pipecolinic acid, found in the iron-deficient plants, is also present with manganese deficiency.

IV. Discussion

The results obtained with the plants grown in full nutrient are of some interest. The range of compounds identified was similar to that found by Pearse and Novellie (1953) in tobacco leaves, by McKee and Urbach (1953) in apple leaves, and by Richards and Berner (1954) in barley leaves. The identification of citrulline and ethanolamine was unexpected, although the presence of citrulline as a major constituent of the amino nitrogen fraction in alder has been reported by Virtanen and Miettinen (1953). The presence of ethanolamine in plants has also been recorded by Woolley (1943). It is significant that cystine, methionine, tryptophan, and tyrosine were not identified, although tests with pure substances showed that they were not lost during extraction. The absence of these compounds from the free amino acid pool of plants has been recorded by Steward *et al.* (1954).

Certain amino acids have only been found in deficient plants, β-alanine in those lacking molybdenum, zinc, or copper, and pipecolinic acid in those deficient in manganese or iron. Certain other amino acids, although present in the controls, have not been found in the free pool of deficient plants. Phenylalanine was characteristically absent from the free amino acid fraction of copper-deficient plants although it was present in every other case. Histidine and lysine were not present in copper-, manganese-, and molybdenum-deficient plants, and lysine was not detectable in the iron-deficient material.

These qualitative changes were accompanied by extensive quantitative changes in the composition of the free amino acid fraction in the case of every deficiency investigated. However, the full significance of these differences is difficult to estimate from the present series of data, as it is recognized that the basis of comparison used here carries an important limitation.

Concentrations of different plant constituents change during ontogeny, and comparison of plants on a basis of temporal age may therefore be misleading if development proceeds at different rates within the same time interval. Physiological age might be a more satisfactory basis of comparison. The identification of this, however, presents considerable difficulty and it may be questioned whether a plant deficient in one essential element can at any time be regarded as being the same physiological age as another which has not been grown with a corresponding restraint.

The differences due to development or physiological age are likely to be least extensive in the early phases of growth, and the basis of comparison that has been used here is therefore likely to be the least misleading, since the plants were young when harvested. The relatively close agreement, when the results were expressed on a concentration basis, i.e. μg of amino acid per mg dry weight, between the results for the two groups of control plants of differing age, weight, and development indicates that the concentration drifts with time over the range of the present experiments were not large.

Within their limitations the present series of data do show that micronutrients have a profound effect on amino acid metabolism. The most important of these is the general accumulation of free amino nitrogen compounds under all deficiencies tested except that of molybdenum. This result is in agreement with the recent work of Steinberg, Specht, and Roller (1955) with respect to all deficiencies except that of molybdenum. With regard to the molybdenum effect, the data assembled here agree with the earlier observations of Hewitt *et al.* (1949) and of Spencer and Wood (1954).

Molybdenum is the only element of those examined that is known to be directly involved in the nitrogen metabolism of plants (Nicholas, Nason, and McElroy 1954), and it is perhaps significant that it was only a deficiency of this element which led to a reduction in the level of free amino acids and amides.

There appear to be two groups within the other four deficiencies tested. Firstly, zinc and iron deficiencies which brought about large accumulations of asparagine and glutamine, and significant increases in the levels of many of the free amino acids. Secondly, deficiencies of copper and manganese which increased the free amino acid levels but had little effect on the total amide levels.

These differences may be due to variation in the stage of deficiency. This is improbable, however, since the symptoms in all cases were severe. In this connection it is of some significance that Prianischnikov (1922) showed that amides accumulate in plants in which proteins are being hydrolysed, and that Gregory (1937) has shown that amides are formed in the lower leaves of potassium-deficient barley at the expense of proteins which are degraded. Thus the amide accumulation noted here may correspond to an accentuated degradation of protein in the absence of iron and zinc.

The present series of results are of some significance in relation to the general character of the changes in the amino acid pool induced by different mineral deficiencies. Richards and Berner (1954) found that the general composition of the free amino acid fraction in leaves of barley does not change substantially with changes in nutrition. The results of this investigation are clearly consistent with this general conclusion. Except for relatively small anomalies the array of amino acids remains the same whatever the nutrient treatment. The suppressions of histidine with copper, manganese, iron, and molybdenum deficiency, of phenylalanine with copper, manganese, and molybdenum deficiency are of little significance, since the concentrations of these amino acids in the control plants are low. Again, the emergence of β-alanine with zinc, copper, and molybdenum deficiency, and of pipecolinic acid with manganese and iron deficiency may be disregarded since the concentration of these is also low. Every major constituent in the pool of control plants is present in that of deficient plants. At the same time the quantitative data show that, while the general array of amino acids does not change with nutrient conditions, the quantitative relations of the amino acid pool do change very considerably.

Two aspects of the situation may be distinguished. Firstly, in terms of unit dry weight there may be either a general increase or a general decrease in the concentrations of all amino acids. Secondly, the extent of change may differ with each amino acid. For instance, with zinc deficiency in which there is a general increase in amino acid concentration the comparison with the control shows 10-fold increases with asparagine, glutamine, histidine, and phenylalanine, but only two- to five-fold increases with aspartic acid, serine, threonine, valine, and proline. The position is similar with every other deficiency.

It may be noted that the quantitative changes, while different with each deficiency, are in certain cases similar. Thus the effects of zinc deficiency are similar to those of iron deficiency with respect to the accumulation of amides and aspartic acid. This, and the circumstance that these deficiencies are not characterized by the suppression of any major amino acid or by the accumulation of any unusual substance, limits very considerably the possibility of using the composition of the amino acid pool as a diagnostic method.

Finally, the data of this amino acid survey of the one plant species grown under a series of nutritional conditions is important in relation to the many amino acid surveys made of other plant species (reviewed by Steward, Zacharius, and Pollard 1955). In general, no two plant species have identical free amino acid pools and usually the differences are quite large. Certain amino acids have been found in only a restricted few plant species, often very widely separated systematically, and additionally, compounds which are major components of the free amino acid fraction of one plant are often barely detectable in another plant species.

The present data suggest that, when a particular amino acid does not accumulate, this does not necessarily indicate that the catalytic systems required for synthesis are not available. In this investigation tryptophan and cystine have not been identified on the chromatograms. These acids, however, are certainly present in the proteins of tomato plants and the enzyme systems involved in their metabolism are therefore certainly available. Secondly, the work of Nason, Oldewurtel, and Propst (1952) has shown that deficiencies of micronutrient elements in plants, in general, bring about the retardation of certain enzyme-catalysed reactions and the acceleration of others. The nutrient deficiency usually does not involve the complete suppression of any system. Thus when phenylalanine is not developed in the absence of copper this may indicate a change in the rate of a reaction involved in the metabolism of this substance. With copper deficiency the appropriate catalytic system is still available, although the rate of some reaction which depends on it is altered. Again when a substance accumulates, as β-alanine does with zinc deficiency, this does not imply a corresponding change in the catalytic system. It is probably due to a change in the concentration of some other reactant in a reaction chain, and it is probable that with respect to β-alanine the catalytic system is the same in the plants supplied with full and with deficient nutrients.

Clearly, the accumulation or depression of any particular substance does not necessarily correspond to changes in the catalytic system. Since this is the case, then it is clear that with any particular species the composition of the amino acid pool will only be characteristic of that species with a particular set of circumstances. It is certainly possible that an amino acid that is identified under certain temperature and nutritional conditions will not be found with different circumstances that do not affect the catalytic system but which influence the rates of the reactions which depend on this system.

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VI. References

Acher, R., Fromageot, C., and Jutisz, M. (1950).—*Biochem. Biophys. Acta* **5**: 81–8.

Bean, R. S. (1942).—Ph. D. Thesis, University of California.

Consden, R., Gordon, A. H., and Martin, A. J. P. (1944).—*Biochem. J.* **38**: 224–32.

Dent, C. E. (1948).—*Biochem. J.* **43**: 169–80.

Eaton, S. V. (1941).—*Plant Physiol.* **7**: 565–95.

Gentry, E. H. R., and Sherrington, L. G. (1950).—*Analyst* **75**: 17–21.

Gregory, F. G. (1937).—*Annu. Rev. Biochem.* **6**: 557–78.

Gregory, F. G., and Sen, P. K. (1937).—*Ann. Bot.* (N.S.) **1**: 521–40.

Hambidge, G. (1941).—"Hunger Signs in Crops." (Amer. Soc. Agron. and Nat. Fert. Assoc.: Washington, U.S.A.)

Hewitt, E. J., Jones, E. W., and Williams, A. H. (1949).—*Nature* **163**: 681–82.

Hoagland, D. R., and Arnon, D. I. (1938).—Circ. Calif. Agric. Exp. Sta. No. 347. 39 pp.

McKee, H. S., and Urbach, G. E. (1953).—*Aust. J. Biol. Sci.* **6**: 369–78.

Nason, A., Oldewurtel, H. A., and Propst, L. M. (1952).—*Arch. Biochem. Biophys.* **38**: 1–13.

Nicholas, D. J. J., Nason, A., McElroy, W. D. (1954).—*J. Biol. Chem.* **207**: 341–51.

Novellie, L. (1950).—*Nature* **166**: 1000.

Pearse, H. L., and Novellie, L. (1953).—*J. Sci. Food Agric.* **4**: 108–12.

Prianischnikov, D. (1922).—*Ber. dtsch. bot. Ges.* **40**: 242–68.

Reed, L. J. (1950).—*J. Biol. Chem.* **183**: 451–8.

Richards, F. J., and Berner, E. (1954).—*Ann. Bot.* (N.S.) **17**: 15–33.

Richards, F. J., and Coleman, R. G. (1952).—*Nature* **170**: 460.

Richards, F. J., and Templeman, W. G. (1936).—*Ann. Bot.* **50**: 367–402.

Sakaguchi, S. (1950).—*Biochem. J.* (*Japan*) **37**: 23–38.

Sanger, F., and Tuppy, H. (1951).—*Biochem. J.* **49**: 463–80.

Spencer, D., and Wood, J. G. (1954).—*Aust. J. Biol. Sci.* **7**: 425–34.

Steinberg, R. A., Bowling, J. D., and McMurtry, J. E. (1950).—*Plant Physiol.* **25**: 279–88.

Steinberg, R. A., Specht, A. W., and Roller, E. M. (1955).—*Plant Physiol.* **30**: 123–29.

Steward, F. C., Wetmore, R. H., Thompson, J. F., and Nitsch, J. P. (1954).—*Amer. J. Bot.* **41**: 123–34.

Steward, F. C., Zacharius, R. M., and Pollard, J. K. (1955).—*Ann. Acad. Aci. fenn.* **A.II.60**: 321–66.

STOUT, P. R., and ARNON, D. I. (1939).—*Amer. J. Bot.* **26**: 144–53.

VIRTANEN, A. I., and MEITTINEN, J. K. (1953).—*Biochem. Biophys. Acta* **12**: 181–87.

WALLACE, T. (1951).—"Diagnosis of Mineral Deficiencies in Plants by Visual Symptoms." (H.M.S.O.: London.)

WELLINGTON, E. F. (1952).—*Canad. J. Chem.* **30**: 581–91.

WILLIAMS, R. J., and KIRBY, H. (1948).—*Science* **107**: 481–3.

WOODWARD, C. C., and RABIDEAU, G. S. (1953).—*Plant Physiol.* **28**: 535–8.

WOOLLEY, D. W. (1943).—*J. Biol. Chem.* **147**: 581–91.

ABSORPTION OF WATER FROM ATMOSPHERES OF DIFFERENT HUMIDITY AND ITS TRANSPORT THROUGH PLANTS

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Summary

An intact plant was arranged so that the top and roots were sealed in separate flasks. In each flask the vapour pressure was maintained at a predetermined level so that a diffusion gradient favouring water movement either from roots to top or from top to roots was established.

With 1-year-old seedlings of short leaf pine (*Pinus echinata*) the absorption of moisture from one flask and its transfer through the plant to the other has been demonstrated, regardless of whether the gradient favoured movement in the direction of normal transpiration (positive transport) or in the opposite direction (negative transport). Absorption occurred from both saturated and unsaturated atmospheres.

The relevance of the results to the absorption of dew and of moisture from unsaturated atmospheres is discussed.

I. Introduction

In recent years a number of investigations have been made concerning the absorption of water by the leaves of plants, and its transfer through the plant to the medium in which the roots are located. In particular the work of Breazeale, McGeorge, and Breazeale (1950, 1951), Haines (1952), Breazeale and McGeorge (1953*a*, 1953*b*), and Duvdevani (unpublished data) can be cited as offering strong evidence for the existence of such movement. In general, these investigators placed the tops of the plants under study in a fogged, supersaturated atmosphere and observed accumulation of water in the vessel containing the roots. The fogging was achieved by the continuous operation of an atomizer or fine sprayer, so that droplets of water formed on the plants. Although the results of these investigations aroused some controversy, it would seem, as Haines (1952) pointed out, that the absorption and movement of water through the plant under these conditions was to be expected as a gradient of increasing diffusion pressure deficit† (DPD) existed from the environment surrounding the leaves to the environment surrounding the roots. The significance of these studies in relation to the absorption of dew by plants under natural conditions has been stressed, in particular by Duvdevani (unpublished data).

Stone, Went, and Young (1950) contributed to the subject in an experiment which demonstrated absorption of water from an unsaturated atmosphere. In their investigation, a small "Plexiglass" chamber was sealed around the top of 2-year-old Coulter pine seedlings, and water-saturated air blown into the chamber. The plants tested were growing in very dry soil, and to induce further water stress they had been exposed to forced ventilation for 48 hr prior to the tests. They were left in the chambers for 24 hr, at the end of which time the relative humidity, as determined by

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† Using the terminology of Meyer (1945).

a humidity sensor inside the chamber, was recorded. In most of the tests the humidity was reduced to a level equivalent to a DPD of more than 150 atm, and in one case to 230 atm. The authors concluded that DPD's of this magnitude existed in the plant and were responsible for the uptake of moisture. They did not measure soil moisture, and so could not determine whether or not the moisture was transported through the plant to the soil.

Theoretically it would be expected that the water would move into the soil as long as the DPD of the soil water was higher than the DPD of the water in the plant, and that transport of water from the atmosphere to the soil would occur whenever a DPD gradient favoured such transport.

In order to take this investigation one stage further, equipment was made up so that the top and roots of an intact plant could be placed independently in atmospheres of known vapour pressure (and hence of known DPD). By establishing DPD gradients at desired levels it was hoped to demonstrate transport of water through the plant in both directions.

II. Apparatus and Methods

The apparatus consisted of two cylindrical flasks, each 8 in. long and $1\frac{1}{2}$ in. in diameter, joined by a ground glass central junction so that the top projected into one flask and the roots into the other (see Fig. 1). The inner walls of the flasks were lined with filter paper which dipped into a reservoir of sodium chloride solution of known concentration at the bottom. A baffle midway along the bottom of each flask prevented undue movement of the solution during manipulation.

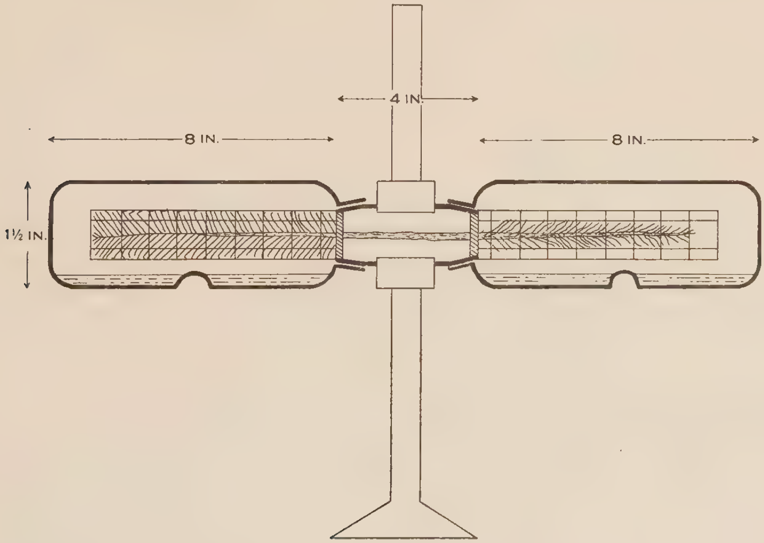

Fig. 1.—Diagrammatic view of apparatus. This whole unit was placed in a constant temperature water-bath for the determinations.

The plant was prepared by washing away the soil from around the roots (after the pot containing the plant had been saturated for 24 hr to facilitate washing). It was then sealed in the central tube with "Permagum"* and "Tackiwax"*, the roots dried as completely as possible with filter paper, and the roots and leaves wrapped loosely with an $\frac{1}{8}$-in. screen mesh to prevent the plant material from dipping into the solution or touching the sides of the flasks.

Prior to insertion, approximately 50 ml of sodium chloride solution of the desired concentration were added to each flask which was then stoppered, weighed to 0·01 g, and allowed to equilibrate for 24 hr in a constant temperature water-bath. As soon as the central unit containing the plant was ready, the flasks were removed from the bath, unstoppered, the plant material inserted, and the apparatus replaced in the bath. Loss of weight by the flasks during the insertion of the plant material did not exceed 0·02 g. At the conclusion of the determination the apparatus was removed from the bath, the plant material extracted and the flasks restoppered, dried, and reweighed to 0·01 g.

The plant was placed in nutrient solution for several days to make sure that death had not occurred during the determination. Change in weight of the flasks was used to determine the extent of water transfer from one flask to the other. During the transfer, loss of turgor in the plant occurred, and this was reflected in a greater gain by one flask than loss by the other. The difference between these amounts represented the degree of turgor loss. Data obtained by direct weighings of the central plant unit before and after each determination were used in the first few experiments to check the results from the flask weights, and to exclude the possibility of leaks in the system. This procedure was found to be unnecessary and was discontinued. No special lighting was provided for the tops of the plants. They received intermittent light from an incandescent light bulb, which comprised the heat source for the water-bath, and diffuse light from normal room illumination.

The constant temperature water-bath used in the experiments was held at 25·0°C and at this temperature was maintained constant to ±0·001°C. Temperature control of this order was very important to minimize the possibility of condensation of water on the plant surfaces. All the experiments were conducted in a constant temperature room, which made the attainment of this degree of temperature control much easier.

III. Results and Discussion

With this apparatus and procedure, two series of experiments were conducted: one with gradients favouring normal transpiration, referred to in this paper as positive transport, and the other with gradients favouring the movement of water in the opposite direction, referred to as negative transport. Two treatments were imposed in each series, the first involving a DPD gradient from 0 atm in one flask to 60 atm in the other, and the second involving a gradient from 15 atm to 60 atm. The former treatment was intended to confirm the results of the investigators who have worked in fogged atmospheres, except that in this case no droplets of water were present; and the latter was intended to extend the work of Stone, Went, and Young (1950) to cover water movement from the aerial environment through the plant to the root environment.

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* Trade names for commercially available sealing compounds.

The plant material used was short leaf pine (*Pinus echinata*) of which a great number of 1-year-old seedlings of uniform size were available. Different plants were used in each experiment, and at insertion the weight of most of the plants was approximately 8 g. The standard period for the experiments was 7 days.

The data obtained from a series of 17 experiments are given in Tables 1 and 2. It is at once evident that both positive transport and negative transport have been demonstrated depending on the direction of the DPD gradient.

Table 1

TRANSPORT OBSERVED WHEN GRADIENT FAVOURED POSITIVE TRANSPORT

Wt. of Plant at Insertion (to nearest g)	DPD of Vapour (atm)		Change in Wt. of Flask (g)		Change in Wt. of Plant (by subtraction) (g)
	Roots	Tops	Roots	Tops	
12*	0	60	−4·81	+5·38	−0·57
8	0	60	−2·61	+2·84	−0·23
8	0	60	−2·88	+3·28	−0·40
8	0	60	−3·28	+3·81	−0·53
Means:	0	60	−2·9	+3·3	−0·4
8	15	60	−1·21	+2·29	−1·08
8	15	60	−1·28	+2·66	−1·38
8	15	60	−1·39	+2·42	−1·03
Means:	15	60	−1·3	+2·5	−1·2

* Results for this plant not included in means.

From Table 1 it is evident that appreciable amounts of water were transferred in both the 0–60 atm and 15–60 atm treatments. Total gain by the 60-atm flask was greater in the former case, as was to be expected from the relative steepness of the gradient. Loss from the 0-atm flask was, however, much greater than from the 15-atm flask and there was an associated decrease in the weight of the plant in the latter treatment. This can be explained when it is realized that the plants at insertion were turgid, and some dehydration had to occur in them before the DPD gradient could develop through the whole system. Thus, in the 0–60 atm treatment evaporation from the leaves of the plant into the 60-atm flask quickly resulted in the development of a DPD of several atmospheres in the plant and so enabled absorption of water through the roots from the 0-atm flask. In the 15–60 atm treatment, however, a DPD in excess of 15 atm had to develop in the plant before a gradient favouring absorption from the 15-atm flask existed. Hence the time taken for the development of this gradient would be expected to markedly reduce the time during which absorption from the 15-atm flask occurred, and hence the amount of total transfer in the 7-day test period.

In Table 2, results of the experiments in which the direction of the DPD gradient favoured negative transport are given. In this case the gains in weight by the 60-atm flasks were about two-thirds as great as in the former series of experiments, and the order of the gain in the 60–0 atm treatment was about one-third greater than in the 60–15 atm treatment.

Although no quantitive data on leaf and root surface areas were obtained, the order of difference in weight gain by the 60-atm flasks in the two series of experiments suggests that a smaller evaporating surface of the roots may have been of importance in this connection. Within the negative transport series the differences in gains by the 60-atm flasks would again seem to be mainly a result of the relative steepness of the gradients.

TABLE 2

TRANSPORT OBSERVED WHEN GRADIENT FAVOURED NEGATIVE TRANSPORT

Wt. of Plant at Insertion (to nearest g)	DPD of Vapour (atm)		Change in Wt. of Flask (g)		Change in Wt. of Plant (by subtraction) (g)
	Roots	Tops	Roots	Tops	
8	60	0	+2·12	−0·20	−1·92
8	60	0	+2·52	−0·63	−1·89
8	60	0	+3·54	−0·88	−2·66
8	60	0	+2·68	−0·50	−2·18
Means:	60	0	+2·7	−0·5	−2·2
5*	60	15	+0·92	−0·12	−0·80
8	60	15	+1·70	−0·37	−1·33
8	60	15	+2·07	−0·43	−1·64
8	60	15	+1·82	−0·44	−1·38
8	60	15	+1·92	−0·43	−1·49
8	60	15	+1·77	−0·28	−1·49
Means:	60	15	+1·9	−0·4	−1·5

* Results for this plant not included in means.

From Table 2 it can be seen that the losses by the 0- and 15-atm flasks were much less than in the positive transport series, and the loss of turgor by the plants during the experiments was much more pronounced. Whereas in the positive transport series, dehydration of the plant only proceeded until a DPD developed of sufficient magnitude to enable absorption of water through the roots, in this series it appears that the plant as a whole was dehydrated until a DPD of the order of 60 atm was developed.* This indicates that the steepest part of the gradient was not at the root surface, but at the leaf surface. In normal transpiration (and in the positive transport series reported above), the steepest part of the gradient is at the leaf surface and it would be expected that the opposite would be true under conditions favouring negative transport. That this does not apply suggests that the cutinized epidermis of a leaf is much less permeable to water than the suberized epidermis of a root, at least in the seedlings used in this study.

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* Confirmatory evidence on this point has been obtained by the author (unpublished data) from a separate experiment. With similar seedlings to those used in this investigation the loss of weight from a turgid plant dehydrated to 60 atm was of the same order as that observed here.

The fact that the whole plant had to be dehydrated to approximately 60 atm before a gradient existed favouring absorption through the leaves explains the relatively small quantities of water absorbed from the 0- and 15-atm flasks in these treatments. Much of the period of the experiment would have been occupied with the development within the plant of a DPD high enough to cause absorption, and the time available for absorption would have been proportionately reduced. Had plants already dehydrated to 60 atm been used it could be expected that loss in the low tension flasks would be of the same order as gain by the high tension flasks. In practice, attempts to introduce dehydrated plants failed because of the degree of root mortality which followed rapid desiccation during the preparation of the material.

In conclusion it may be stated that these experiments have demonstrated that water has been absorbed from one flask and transported through the plant to the other flask, along gradients of DPD, regardless of whether the gradient has favoured positive transport or negative transport. In both cases absorption has occurred not only from an atmosphere of 100 per cent. relative humidity, but also from an unsaturated atmosphere.

This mechanism may have some significance in nature. In the first place it lends support to the hypothesis (put forward by the investigators cited above) that dew can be absorbed by plants, because in all but very wet soils, gradients favouring absorption would exist. Secondly it indicates that moisture may be absorbed from an unsaturated atmosphere so long as there is a favourable gradient. Such conditions can be expected to occur in areas of suboptimal rainfall, and particularly in semi-arid and arid regions, where humidity levels reach quite high values at night, even if dews do not occur. In these regions plants persist for protracted periods in very dry soil, and the DPD of the soil moisture may be of the order of several hundred atmospheres (Burr 1914; Alway, McDole, and Trumbull 1919). It is conceivable that DPD gradients favouring negative transport could develop, and water may move along these gradients from the atmosphere to the plant, and possibly through the plant to the soil. To what extent such transport could benefit plants is a matter for physiological study. The actual amounts of water involved under natural conditions have yet to be determined but it seems that even small amounts of additional water would be of benefit to plants growing in areas of low rainfall.

IV. ACKNOWLEDGMENTS

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V. References

Alway, F. J., McDole, G. R., and Trumbull, R. S. (1919).—Relation of minimum moisture content of subsoil of prairies to hygroscopic coefficient. *Bot. Gaz.* **67**: 185–207.

Breazeale, E. L., and McGeorge, W. T. (1953*a*).—Exudation pressure in roots of tomato plants under humid conditions. *Soil. Sci.* **75**: 293–8.

Breazeale, E. L., and McGeorge, W. T. (1953*b*).—Influence of atmospheric humidity on root growth. *Soil Sci.* **76**: 361–5.

Breazeale, E. L., McGeorge, W. T., and Breazeale, J. F. (1950).—Moisture absorption by plants growing in an atmosphere of high humidity. *Plant Physiol.* **25**: 413–19.

Breazeale, E. L., McGeorge, W. T., and Breazeale, J. F. (1951).—Water absorption and transpiration by leaves. *Soil Sci.* **72**: 239–44.

Burr, W. W. (1914). —The storage and use of soil moistures. Res. Bull. Neb. Agric. Exp. Sta. No. 5.

Haines, F. M. (1952).—The absorption of water by leaves in an atmosphere of high humidity. *J. Exp. Bot.* **3**: 95–8.

Meyer, B. S. (1945).—A critical evaluation of the terminology of diffusive phenomena. *Plant Physiol.* **20**: 142–64.

Stone, E. C., Went, F. W., and Young, C. L. (1950).—Water absorption from the atmosphere by plants growing in dry soil. *Science* **111**: 546–8.

STUDIES ON THE DEVELOPMENT OF WOOL FOLLICLES IN TISSUE CULTURE

By Margaret H. Hardy* and A. G. Lyne*

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Summary

Explants of skin from four sheep foetuses of different ages have been cultivated for periods of up to 38 days in a medium of fowl plasma and chicken embryo extract. The development of wool follicles of different types has been studied both in these living explants and in serial sections from them.

Explants from a 70-day foetus showed the complete development of primary wool follicles *in vitro* from epidermal plugs to fully differentiated follicles with emerging wool fibres. The histology of these follicles was normal and they developed at about the same rate as in the foetus *in utero*. Sebaceous glands of normal size and structure were differentiated *in vitro*, and, for the first time in any species, the formation of rudimentary sudoriferous glands *in vitro* has been reported.

In explants from an 80-day foetus, primary wool follicles which were at an early stage of development produced keratinized wool fibres at a slightly faster rate than in the foetus *in utero*, but secondary follicles did not form. Sebaceous glands were formed and sudoriferous glands underwent normal differentiation.

The primary follicles in explants from a 98-day foetus produced many large wool fibres but development was slower than in the foetus. There was some evidence for the formation of new secondary follicles *in vitro*.

There was little or no development in the wool follicles in explants from a 125-day foetus.

The potential value of this method for studying mechanical, nutritional, or hormonal influences on the initiation and early development of wool follicles and the production of wool is indicated.

I. Introduction

The study of living and growing organs in tissue culture has contributed much to the understanding of their development and function (Fell 1940, 1951). Hair follicles are suitable for cultivation because by virtue of their small size they might be expected to grow and function for some time without a blood circulation, and also their day-to-day changes in structure and output of hair should be more easily observable in explants than by any means available with the intact animal. Tissue culture has already been applied to the hair follicles of pre-natal guinea pigs (Strangeways 1931) and rats (Murray 1933) and has proved useful in studies of hair development in mice (Hardy 1949, 1951; Davidson and Hardy 1952). However, the sheep is a more suitable subject than any of the rodents for investigating some problems such as the formation of hair follicle groups (Carter 1943).

Only one reference has been found to the cultivation of tissues from the sheep. Thomas *et al.* (1948) removed large foetuses of cattle and sheep from the uterus and

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maintained them by perfusion for more than 2 days, but no differentiation of tissues was reported. The aims of the present study, a short account of which has already been published (Hardy and Lyne 1956*c*), were firstly to find a suitable technique for cultivating the skin of foetal sheep, secondly to observe the formation of individual wool follicles of different types, and thirdly to observe the formation of follicle groups and the effect of cultivation thereon. Such information is a prerequisite to assessing the potential value of tissue culture as a tool in investigations of the effect of hormonal, nutritional, or environmental factors on wool production.

II. Material and Methods

Skin for tissue culture was obtained in a fresh condition from four sheep foetuses. Two of these, believed to be Merino, were obtained *in utero* from the State Abattoir and Meat Works, Homebush, N.S.W. Their ages were estimated by the method previously indicated (Hardy and Lyne 1956*d*) to be 70 and 80 days of gestation. Skin samples were also obtained by biopsy, during intra-uterine operation, from two female foetuses of fine-woolled Merino (non-Peppin) parentage. The ages of the latter foetuses were known from mating records to be 98 and 125 days. Both the 80- and the 98-day foetuses were members of a pair of twins.

A series of hanging-drop cultures was prepared from skin from the midside of the 70-, 80-, and 98-day foetuses and from the shoulder of the 125-day foetus. The method of cultivation was similar to that used for the mouse (Hardy 1949). With the aid of a low-power binocular microscope the original skin sample was cut into small pieces with cataract knives. On the basis of a comparison, to be described in Section III, of the effectiveness of different techniques and media on explants from the 70-day foetus, all other cultures were prepared in the following way. Each explant consisted of the full thickness of skin with an epidermal surface area of 1–2 sq. mm, and was placed either on the surface or inside the medium, usually with the dermis nearest to the coverslip. The medium consisted of fowl plasma and chicken embryo extract in ratios ranging from 3:1 to 1:1. The extract was prepared from chicken embryos 10–12 days old and the same or twice the volume of Tyrode solution. The explants were transferred to fresh medium every 2–6 days as required, and a few large explants were divided at the time of transfer. All explants were examined daily under the microscope and many important features were sketched. In each of the four experiments some explants were fixed every few days to give a record of the progress made during cultivation, and the remainder were fixed when no further progress was discernible. All explants were fixed in Zenker's fluid with 3 per cent. acetic acid. Most were sectioned serially at 8 μ and stained with haemalum, eosin, and picric acid, and some were examined as whole mounts after staining with carmalum, borax carmine, or safranin. The 70- and 98-day control skin samples were fixed in Zenker with 3 per cent. acetic acid and the other control samples in 5 per cent. formalin. All control skin was sectioned serially at 7 or 8 μ parallel to the skin surface and also in the direction of the long axis of the follicles.

The terms and symbols used for follicle types are those proposed by the authors (Hardy and Lyne 1956*a*) and explained in a recent paper (Hardy and Lyne 1956*d*).

III. Results

The present authors have recently re-examined the development of different types of wool follicles in the Merino foetus (Hardy and Lyne 1956*b*, 1956*d*). Eight stages of follicle development in mammals (*F* stages) have been defined and their features in primary (*P*) and secondary (*S*) wool follicles have been described. These were then related to Carter's (1943) 18 stages of follicle group development (*G* stages) in the Merino. This study formed the basis for comparing follicle development in tissue culture with that in the foetus. Only an average rate of follicle development in the foetus is available (Hardy and Lyne 1956*d*), but some idea of the extent of variation between foetuses can be obtained from Carter and Hardy (1947) and Hardy and Lyne (1956*d*). There is no information on the rate of development in the foetus from day to day to compare with the observations of this kind which are possible in tissue culture.

Table 1

SUMMARY OF EXPERIMENTS

Age of Foetus (days)	No. of Explants				Maximum Period of Cultivation (days)	Most Advanced *PCX* Follicle Stage Developed		
	Total	Discarded*	Successfully Cultivated	Showing Follicle Development†		Initial	*in vitro*	*in vivo*
70	29‡	1	28	28	38	*F*1	*F*8	*F*8
80	20	1	19	19	16	*F*2*a*	*F*6	*F*4
98	44	0	44	44	16	*F*6	*F*8	*F*8
125	42	1	41	0	11	*F*8	*F*8	*F*8

* Due to infection or failure to survive explantation.

† Excluding development of hair canals.

‡ Omitting 12 half-thickness explants.

Some of the differences between the results of the four tissue culture experiments are indicated in Table 1. Follicle development *in vitro* was observed in nearly all explants in the first three experiments but in none in the fourth experiment. The maximum period of cultivation, which was approximately the same as the period during which progress in wool follicle development was observed, was long in the first experiment but shorter in the second and third. The number of stages which the first-formed central primary (*PCX*) follicles passed through during cultivation was progressively smaller with increasing age of the foetus. This was partly due to the fact that stage *F*8, the emergence of a wool fibre, was the last stage recorded. However, the growth rate of fibres *in vitro* during stage *F*8 was lower than that *in vivo*, so that it is true that overall progress was less in the older foetuses. The last two columns in Table 1 show that in all experiments the stage reached *in vitro* was as advanced as the one at the corresponding age *in vivo*, and in explants from the 80-day foetus it was more advanced.

Another difference between the experiments, not expressed in Table 1, was that the older the foetal skin, the more frequently did degenerative changes occur after a period of cultivation.

(*a*) 70-*day Foetus*

(i) *Structure at Explantation.*—The epidermis consisted of a stratum germinativum of one cell layer, a stratum spinosum of two to three layers, and a periderm of one layer. The dermis consisted of a network of fibroblasts penetrated by small blood vessels. Wool follicle group development was at stage *G*2, with *PCX* and a few of the later-formed central primary (*PCY*) follicles present. The *PCX* follicles were at an advanced stage *F*1, not quite up to *F*2, (Plate 1, Figs. 1 and 2), and the *PCY* follicles were at a very early stage *F*1.

(ii) *Behaviour of Explants.*—Although most explants at first remained fairly flat on the surface of or inside the medium, after about 77 days (age of foetus+age of explant) they showed a tendency to curl up with the epidermis inside and the dermis spreading around it. The follicles therefore extended in all directions into the medium, and wool fibres grew towards the centre of the explant. This orientation, similar to that which develops in skin explants from embryonic mice (Hardy 1949, Text-fig. 2), has the advantage that follicles around the edge of the explant can be seen very clearly in the living state. The epidermis underwent normal differentiation, but usually keratinization took place about 10–15 days earlier than in the foetus, and a thick central mass of cornified cells was formed in some of the older explants. The dermal tissue proliferated and some connective tissue fibres were formed during cultivation. A healthy outgrowth of fibroblasts appeared at 71 or 72 days and new fibroblast outgrowth continued throughout the period of cultivation. After about 80 days there was frequently an epithelial outgrowth also.

(iii) *Effect of Treatments.*—In this first experiment with foetal sheep skin, the technique described in the preceding section was compared with a number of variations from it. The plasma of the medium was fowl plasma, sheep plasma, or a mixture of the two in equal quantities. Some explants were placed horizontally, i.e. with the skin surface parallel to the surface of the medium and the dermis nearest to the coverslip, or with the epidermis nearest to the coverslip, and others were placed vertically, i.e. with the larger cut edges parallel to the surface of the medium and the smaller edges perpendicular to the medium. The full thickness of skin was included in some explants, and others had the deeper half of the dermis removed with cataract knives. One large full-thickness explant with a surface area of about 12 sq.mm was prepared.

Explants in sheep plasma seldom showed any outgrowth before 75 or 76 days, while those in fowl plasma or fowl+sheep plasma had a good outgrowth of fibroblasts at 71 or 72 days. At 80 days explants in sheep plasma still had less outgrowth than those in the other two groups, but after 82 or 83 days there was no difference. Up to 87 or 88 days no differences in health or follicle development could be attributed to the type of plasma, but at this time all remaining explants in fowl+sheep and sheep plasma were fixed because of signs of early degeneration, while some of those in fowl plasma continued until 101 or 108 days with little degeneration. No important differences were noted between explants placed on the surface and those within the medium, those with the dermis nearest the coverslip and those with the epidermis nearest, or those oriented horizontally and vertically. Owing to their tendency to curl up, most explants eventually assumed much the same form irrespective of their original orientation. Explants of half-thickness skin were much less healthy than those of full-thickness skin although the outgrowth from them was similar. Full-thickness explants had increased in volume at 74 days, but a decrease in volume of half-thickness explants was noted at 72 and 74 days. More than half of the half-thickness explants showed degeneration at 74 days and only one of them showed any progress in follicle development, to stage *F*2 at 77 days. The large full-thickness explant progressed normally until 78 days and then underwent central degeneration, presumably due to inadequate diffusion.

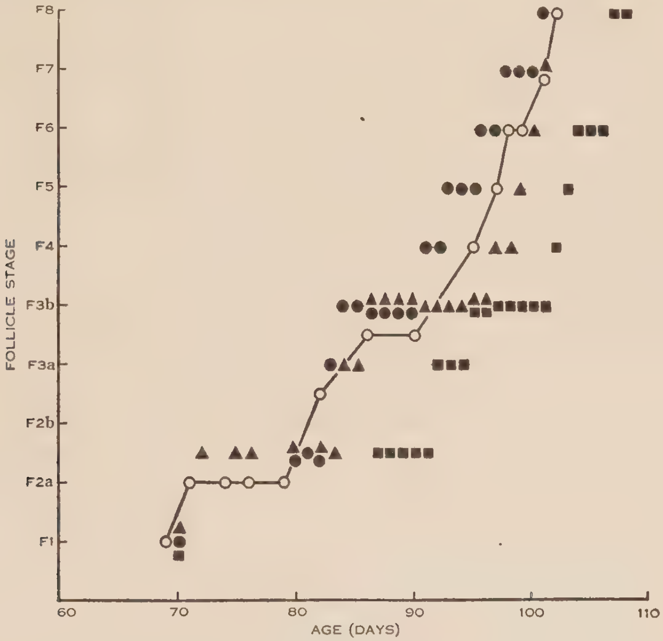

Fig. 1.—The rate of development of the most advanced primary follicles in three explants (●, ▲, ■) from the 70-day foetus compared with the average rate in the most advanced *PCX* follicles in the Merino foetus (○).

(iv) *Progress of* PCX *Follicles*.—A record was made of the most advanced stage of follicle development which could be seen each day in each explant. From the daily identification and sketching of many individual follicles it was concluded that the most advanced follicles in an explant at any time were predominantly the *PCX* follicles originally present. In one of the 25 explants for which complete records were available, the stage *F*1 follicles elongated without reaching stage *F*2 before fixation at 74 days, but in all others new *F* stages appeared *in vitro*. The most advanced follicles observed in explants were frequently at the same stage as in the "average" foetus of the same age, but sometimes slightly ahead or slightly behind. At fixation, for example, 17 explants were at the same stage, four were ahead, and four were behind. Figure 1 illustrates the degree to which follicle development in three explants corresponded with the average rate in the foetus. The variation between explants in the most advanced stage of follicle development after some days of cultivation was greater than the variation between adjacent 1-sq.mm areas on a foetus, but probably much less than the variation between foetuses of the same age (Carter and Hardy 1947).

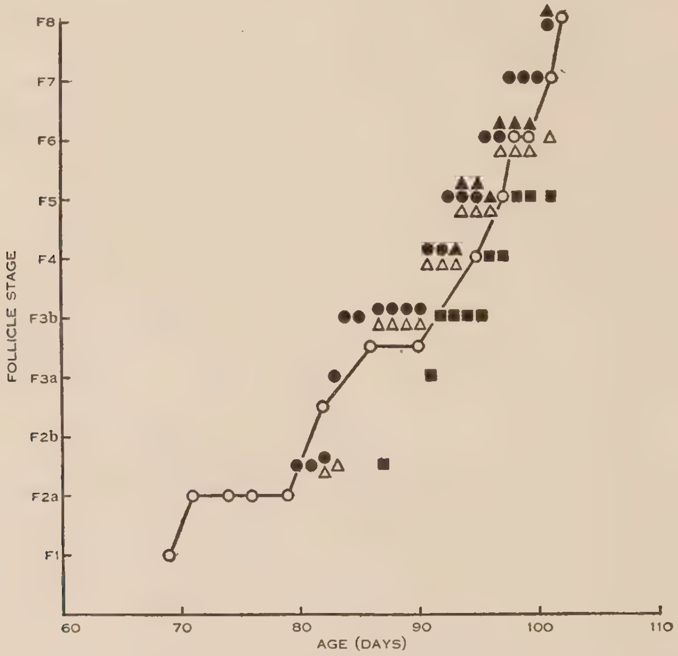

Fig. 2.—The rate of development of four follicles (● = 1, ▲ = 2, ■ = 3, △ = 4) in one explant from the 70-day foetus compared with the average rate in the most advanced *PCX* follicles in the Merino foetus (○).

Figure 2 shows the rate of development of a few individual follicles in one explant. Follicles 1, 2, and 4 are almost certainly *PCX* follicles, but follicle 3 is possibly a *PCY* or a *PLx* (lateral primary associated with *PCX*) follicle. This figure illustrates the similarity between rates of development in early and later *P* follicles within an explant, comparable with that in the foetus (Hardy and Lyne 1956*d*).

(v) *Progress of Other Follicles.*—Information about follicles other than *PCX* was more difficult to obtain. The first new follicles which appeared during cultivation were identified and their development followed for several days. These were similar to the *PCY* follicles already present and appeared in the spaces between the *PCX* follicles. In 12 explants at 71 days the numbers of follicles ranged from nine to 19, with a mean of 12, and at 73 days the numbers ranged from 15 to 24 with a mean of 20, representing a 67 per cent. increase in follicle numbers, about what would be expected to make the final numbers of *PCY* and *PCX* follicles roughly equal. By 80 days the numbers of follicles in these explants had increased by about 100–200 per cent. over the numbers at 71 days, and between 80 and 90 days there were further increases in the numbers of follicles in some explants. In one explant which had 11 *PCX* and three *PCY* follicles at 71 days, 37 follicles were observed at 80 days, 34 of these were at *F*3 by 94 days, and 12 were at *F*4 by 99 days. In another explant only one follicle was seen at 71 days and five at 72 days, but at 108 days 19 keratinized hairs were present.

From the above and from similar evidence the following conclusions were drawn. The *PCY* follicles present at 70 days continued their development, some to *F*6 or *F*8. New *PCY* follicles appeared at about the normal time (71–73 days) and in their typical position, and underwent further development. The additional new follicles which formed between 74 and 80 days were probably equivalent to the *PL* (lateral primary) follicles which appear in the foetus at this time, although in explants they were not arranged in the typical manner on either side of the *PC* (central primary) follicles. The skin surface area of explants did not expand as rapidly as that of the foetus, so that the follicles were tightly packed together and any tendency to grouping was likely to be obscured. Some at least of these "*PL*" follicles developed to stage *F*3 or further. There was no direct evidence for the formation of *S* (secondary) follicles, which begins in the foetus at about 86 days. A few follicles at stages *F*1 and *F*2 were noted in the explants fixed at 101–108 days, but since these particular ones could not be traced back to their origin in the living explants there was no means of determining whether they were new *S* follicles or retarded *P* follicles.

(vi) *Histology*.—All the histological features which distinguish the eight stages of hair follicles in mammals were observed *in vitro*. Many of these are illustrated in Plate 1, Figures 3–6, and in the comparison of Plate 2, Figures 1 and 5, with Plate 2, Figures 2 and 6, respectively. In explants cultivated for a sufficient period many wool fibres with a keratinized cuticle and cortex of normal structure were formed (Plate 1, Fig. 6). Medullated fibres were not found, but it is quite normal for *P* fibres on the midside of Merino foetuses between 100 and 110 days to be non-medullated. Apart from the absence of arrector pili muscles, and from variations in the sebaceous and sudoriferous glands to be described below, only the following minor differences were noted between primary follicles formed in explants and those in the Merino foetus. There was a tendency for the follicles in explants to be shorter in proportion to their width, and for the ental swelling of the outer root sheath to be more conspicuous. In explants the follicles were more frequently curved or twisted, and, because of the central position and precocious keratinization of the epidermis, the wool fibres had difficulty in penetrating it and frequently coiled beneath the surface.

The sebaceous gland rudiments made their first appearance in explants relatively late, when follicles were at stages *F*3*b* or *F*4 (92–102 days) instead of in the latter part of stage *F*2 (82 days) as in the foetus. However, they subsequently developed into glands comparable in size to those in foetuses of corresponding age (Plate 1, Fig. 6), and some were bilobed in the manner typical of those attached to *P* follicles (Plate 2, Figs. 1 and 2). Holocrine secretion from the normally differentiated sebaceous cells was observed (Plate 2, Fig. 4).

Sudoriferous glands appeared late in stage *F*2 (76–83 days) instead of at the beginning of *F*2 (71 days). The gland rudiments were observed in the living state in only five out of 18 explants cultivated after 80 days. Of these five explants, only one showed sudoriferous glands in stained sections after fixation (Plate 1, Fig. 4). The rudiments disappeared from the other living explants after a few days and it was assumed that they had regressed. Three other explants showed sudoriferous gland rudiments in sections. Unlike the sebaceous glands, the sudoriferous glands did not attain a size or degree of development comparable with that in the foetus of 100 days or more, but always remained in a rudimentary state. The most advanced sudoriferous gland found in any explant (at 101 days) was a short blind tube with a small distal lumen, typical in histology though shorter than the gland of a *PC* follicle in the foetus at *F*4 or *F*5 (95–97 days). It was attached to a follicle at *F*7 (Plate 2, Figs. 1 and 3).

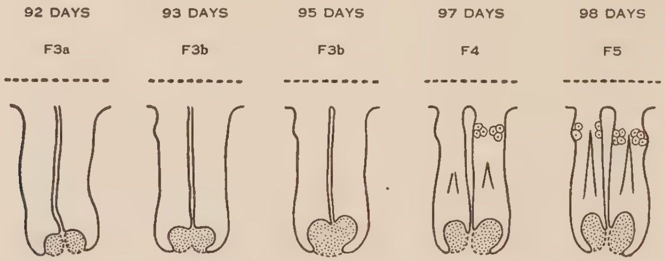

Fig. 3.—Diagram showing the progress from stage *F*3*a* to *F*5 of "twin" follicles in an explant from the 70-day foetus. Note the separation, fusion, and subsequent separation of the dermal papillae.

One unusual arrangement of developing follicles was noted in three of the five explants cultivated after 88 days. Five pairs of "twin" follicles were found with their dermal papillae joining. Three pairs were noted only in sections but the other two pairs were observed also in a living explant from 91 to 101 days (Fig. 3). In one pair the follicles were contiguous along their whole length and in the other, only at the base. The follicles were at stage *F*3*a* when first observed, and by 101 days one pair was at *F*4 and the other at *F*5. The rates of development of the members of a pair were very similar but not always identical.

(*b*) 80-*day Foetus*

(i) *Structure at Explantation.*—The skin contained follicle groups at stage *G*5. Long *PCX* follicles were at stage *F*2*a*, with sudoriferous glands extending to about two-thirds of the depth of the follicles (Plate 3, Fig. 1). The *PCY* follicles were shorter, also at *F*2*a*, with sudoriferous glands extending to about half the depth of the follicles. The *PLx* follicles were still shorter; some were at *F*2*a* with sudoriferous gland rudiments beginning, and others at *F*1. Some *PLy* follicles were at *F*1 and the remainder had not yet formed. This stage of follicle group development was slightly earlier than that in the average Merino foetus at 80 days, which is stage *G*6 with all four types of follicles at stage *F*2*a*.

(ii) *Behaviour of Explants.*—The explants remained flat until about 85 days, and then about half of them began to curl up like those from the 70-day foetus with the dermis surrounding the epidermis (Plate 3, Fig. 3), while the remainder began to curl in the opposite direction with the epidermis surrounding the dermis and obscuring the view of living follicles. There was no appreciable difference between the two groups in the health of explants or the development of follicles. In both groups keratinization of the epidermis began about 10–15 days earlier than in the foetus. There was a good outgrowth of fibroblasts and a small outgrowth of epithelial cells.

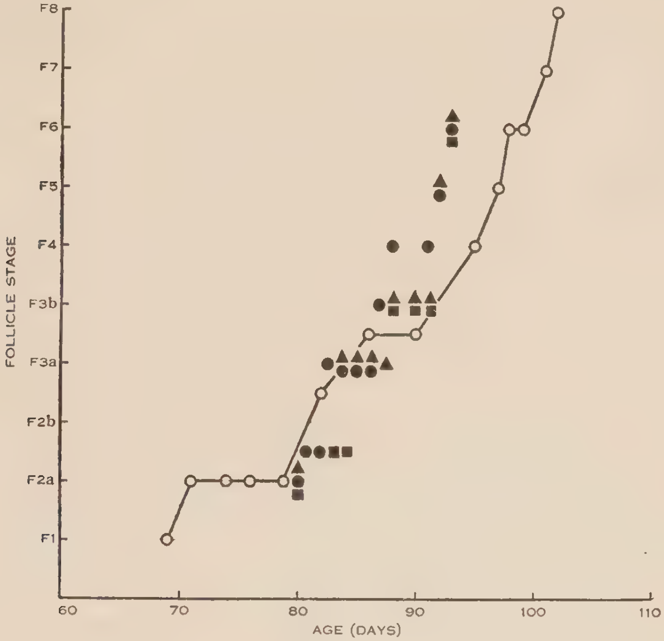

Fig. 4.—The rate of development of the most advanced primary follicles in three explants (●, ▲, ■) from the 80-day foetus compared with the average rate in the most advanced *PCX* follicles in the Merino foetus (○).

(iii) *Progress of Follicles.*—In every explant, some *PCX* follicles underwent further development, to at least stage *F3a* and in some cases to stage *F6*. Unlike the 70-day explants, many of the 80-day explants showed *PCX* follicle development at a more rapid rate than in the foetus. Thus, at fixation, eight explants were ahead, eight were at the expected stage, and three were retarded. The follicles in three explants which reached stage *F6* at 93 days were about 5 days or about 2·5 *F* stages ahead of those *in vivo*. Figure 4 shows their progress compared with that in the foetus. The *PCY* follicles began to develop in the same way as *PCX* follicles but their final stage could not be determined because they could not be distinguished from *PCX* follicles throughout cultivation. However, the use of 80-day explants made possible some observations on the development of *PL* follicles which could be identified from their position on either side of the *PC* follicles. The *PLx* and *PLy* follicles already present were seen to elongate *in vitro*, and some at least reached stages *F3a* and *F3b* by 84 days, much earlier than in the foetus. In five explants during the first few days of cultivation some new *PLy* follicles appeared. The trio arrangement was preserved during cultivation in most of the explants. There was no sign of the formation of *S* follicles.

(iv) *Histology.*—The histology of follicles developed *in vitro* was similar to that of follicles in the 70-day explants (Plate 3, Figs. 2 and 3). Some of the wool fibres were of normal structure but some were incompletely keratinized. As in the 70-day explants, sebaceous gland development was retarded. Gland swellings, some with differentiated sebaceous cells, were first noted in living explants at 87 days or later, in follicles at stage *F3a* or *F3b*. However, sebaceous glands were found in all 19 of the explants fixed between 84 and 96 days.

Sudoriferous gland development was more extensive than in explants from the 70-day foetus. In all explants the glands originally present elongated together with the follicles to which they were attached, and underwent some distal enlargement. In 14 explants at fixation the sudoriferous glands showed a distinct distal lumen. The glands were thus as advanced in their development as the follicles and as sudoriferous glands in foetuses of similar ages. During cultivation a number of new sudoriferous glands appeared in association with *PLx* and *PLy* follicles and underwent some development, although not every *PL* follicle was found to possess a sudoriferous gland at the time of fixation.

In each of three living explants one or more follicles which had become separated from the epidermis were observed. No epidermal tissue other than the follicle wall itself was in contact with these "isolated" follicles, which were completely surrounded by dermis. These follicles were first noted at stage *F2* or *F3a* and progressed to *F3a*, *F4*, and *F6* respectively. One developed a sudoriferous gland rudiment.

(*c*) 98-*day Foetus*

(i) *Structure at Explantation.*—The follicle groups were at stage *G*13, which appears at 99 days *in vivo*. Only a few *PCX* follicles had reached the beginning of stage *F6* and the remainder of the *PCX* and the *PCY* follicles were at *F5*. The *PL* follicles were at *F5*, *F4*, or *F3b*. The original secondary (*SO*) follicles were at *F3a*, *F2*, or *F1*, and a few of the most advanced ones had some differentiated sebaceous cells. On a few of the *SO* follicles at *F3a* and *F2*, a single derived secondary (*SD*) follicle at *F1* was present as a bud. There was usually not more than one *SD* follicle per follicle group, but occasionally two were present.

(ii) *Behaviour of Explants.*—These explants remained flatter during cultivation than those from younger foetuses. There was a healthy outgrowth of fibroblasts and usually a smaller healthy epithelial outgrowth. The explants, however, were less healthy than those from 70- and 80-day foetuses, and degenerative changes took place at the base of many of the larger follicles.

(iii) *Progress and Histology of Follicles.*—During the first few days of cultivation there was an increase in the number of *PCX* follicles at *F6*, and many *PCY* follicles also reached this stage. The longer the period of cultivation, the more likely was an explant to reach *F*8, but the usual rate of progress, unlike that in 70- and 80-day explants, was slower than *in vivo*. Before cultivation the diameter of keratinized fibres varied from 3 to 8 μ (average 4 μ), but after cultivation many fully keratinized, non-medullated fibres of 16–25 μ were found. Other thick fibres in the explants were incompletely keratinized, having areas in which the cuticle was fully keratinized and stainable with picric acid, while the remainder of the fibre was of abnormal structure and stained only with eosin. Healthy sebaceous glands were found with *PC* follicles in this as in other experiments, and the lumina of sudoriferous glands usually enlarged during cultivation. Further progress of *PL* follicles was observed in this experiment, to stage *F*6 or beyond in 12 explants and to *F*5 in most others, but the rate of progress was slower than *in vivo*. Some of the *PL* fibres formed *in vitro* were fully keratinized and others partly keratinized. The sebaceous and sudoriferous glands progressed slightly.

This experiment provided the first opportunity for observations on *S* follicles *in vitro*. It was not possible to determine the extent of development of individual *SO* follicles in 12 whole-mount preparations. In the explants which were sectioned, some *SO* follicles reached stage *F*3*b*, and one possibly *F*4, which indicates a slower rate of development than *in vivo*. However, in skin sections from the foetus used in this experiment (dead) and from its larger twin (alive), when aborted at 118 days, the follicle groups were only at stage *G*16 (instead of *G*17, as in the average foetus), and no *SO* follicles were seen at stages more advanced than *F*3*b*. In some explants the sebaceous glands attached to the *SO* follicles had undergone further development.

Individual *S* follicles were not identified during the course of the experiment, but evidence for any changes during cultivation in the numbers of *S* follicles of either type was sought from follicle counts in sectioned material. The numbers of *S* follicles per follicle group in transverse sections (1) at the mid-sebaceous gland level of *P* follicles, and (2) above the sebaceous glands, in control skin were compared with the numbers at these levels in explants. In control skin the mean number was 2·4 (range 1–3) at level (1) and 4·4 (range 4–5) at level (2). In each of nine explants fixed at 100–111 days there was an increase in the upper limit of the range and usually also in the mean value at level (1). In eight out of nine explants there were corresponding increases at level (2). The greatest increases were found in an explant fixed at 110 days, in which the ranges of counts were 5–8 at level (1) and 5–10 at level (2).

In order to interpret the above results, some reconstructions were made of the *S* follicles in three entire follicle groups from the explant referred to, and in five groups in all from three other explants. Each of these groups contained a number of short *SO* follicles which did not extend as deep as level (1). Some longer *SO* follicles had *SD* follicles budding from them above or occasionally at level (1), but not more than one *SD* was found with any *SO* follicle, and not more than two *SD* follicles were found in any group. In two explants other than those from which reconstructions were made, a few *SO* follicles each with two *SD* follicles were seen, but this was apparently an infrequent occurrence. In one whole mount of an explant fixed at 107 days, follicle groups containing four to eight *S* follicles at level (2) were clearly seen. The numbers of *S* follicles were lower at level (1) and this appeared to be due to the shortness of some *SO* follicles.

It was concluded that in many explants there was development of *S* follicles during cultivation. The increased numbers of *S* per group above sebaceous gland level could be explained by the formation of new *SO* follicles, and the increased number of *S* at the sebaceous gland level could be due to the increase in length of the *SO* follicles originally present and perhaps of some of the new ones. In two explants at least there was probably an increase in the number of *SD* follicles.

(*d*) 125-*day Foetus*

(i) *Structure at Explantation.*—Follicle groups were at stage *G*18, and all *P* and some *SO* follicles were at stage *F*8. Other *SO* follicles were at stages *F*4 to *F*7. The *SD* follicles were numerous, as many as eight *SD* per group being present, and some of them had reached stage *F*3*b* or *F*4.

(ii) *Behaviour of Explants.*—These explants remained fairly flat. The outgrowth of fibroblasts and epithelial cells appeared more slowly than in explants from younger foetuses and was frequently less extensive, but usually appeared healthy. However, the explants showed some degeneration at the base of the larger follicles even at 129 days, and progressive degeneration in the dermis and larger follicles thereafter. The explants fixed at 136 days were very unhealthy.

(iii) *Progress and Histology of Follicles.*—No evidence was found of progress in any of the types of follicles present. The *P* wool fibres did not increase in length, and there was no indication of an increase in the number of keratinized *SO* fibres per group. It was not possible to test whether the number of *SD* follicles had increased.

(*e*) *Development of Follicle Groups in All Experiments*

Although the study of follicle group development was one of the objects of the experiments, Carter's (1943) definitions of group stages were found unsuitable for recording the changes in explants because they referred in turn to differentiation in different types of follicles. Consequently the results of the present experiments have so far been described in terms of the differentiation of each type of follicle separately, but the extent of follicle group development will now be indicated. In the explants from 70-day skin at stage *G*2, stage *G*3 developed as *in vivo* when sudoriferous gland buds appeared with the *PCX* follicles, and new "groups" were initiated by the appearance of new *PCY* follicles. Although the *PCX* follicles continued their development to the stage which they reach at *G*15 *in vivo*, it could not be said that the explants reached even a typical stage *G*4, because the trio arrangement of *P* follicles was not observed, or a stage *G*8, because *S* follicles did not appear. Explants from the 80-day foetus progressed from *G*5 to *G*7, and new *PLy* follicles completed some trio groups, but there was no *G*8, although the development of *PCX* follicles at 93 days corresponded to that of stage *G*12. In the 98-day foetus all follicle types had been initiated, and explants proceeded from stage *G*13 to *G*16 as *in vivo*. The 125-day foetus had already reached the most advanced stage of group development.

IV. DISCUSSION

(*a*) *Technique*

The technique used was found to be reasonably satisfactory for skin from the younger foetuses but unsuitable for the older ones. The surprising superiority, in the first experiment, of plasma from the fowl may be due to mechanical factors, such as the production of a firmer clot. In view of the disappointing results with skin from older foetuses, it would be worth testing the effects on those of semi-homologous, homologous, and autologous media. The finding that the full thickness of dermis was necessary for successful follicle development in skin from the 70-day foetus was also surprising, since the dermis appeared to be histologically homogeneous at this stage, and was very much deeper than necessary to accommodate the extending follicles. In the mouse all that was required for successful follicle development, in addition to epidermis, was a portion of dermal tissue (or subepidermal mesoderm) (Hardy 1949).

(*b*) *Comparison of Sheep and Mouse Hair Follicles* in vitro

The development of the first pelage hair follicles from stage *F*1 to stage *F*8 occupied about 9 or 10 days in the mouse (Hardy 1949) but about 38 days in the sheep, so that tissue cultures from sheep had to be maintained over four times as long to include the same sequence of development. Nevertheless, the maximum degree of progress achieved *in vitro* in a single set of explants was almost as great with the sheep as with the mouse. In mouse skin, follicles initiated *in vitro* progressed to stage *F*8, while in sheep skin, follicles explanted at *F*1 progressed to *F*8. The rate of development of follicles in explants from the mouse was at best very similar to that *in vivo*, and the rate in explants from the 70-day sheep foetus was also similar to that *in vivo*, but the rate in some explants from the 80-day foetus appeared to be significantly faster. The fractions of the total follicle development time occupied by the several stages *in vivo*, or the shapes of the stage-age curves, were different for the two species, and the explants behaved in this respect like the species from which they were taken.

In all essentials, the histogenesis of follicles was normal in explants of both mouse skin and sheep skin. Two abnormalities found in explants of sheep skin are of morphogenetic interest. The "twin" follicles, or structures like them, could perhaps give rise to the multiple fibres of sheep (Auber and Ryder 1956) or other species (Pinkus 1951), the origin of which has not been studied. Moreover, their orientation suggests that the influence of dermal papilla cells on hair matrix cells, in palisade formation, for example, may extend from one follicle to the next. The occurrence of "isolated" follicles indicates that, at certain stages at least, the differentiation of follicles does not require continuity with the epidermis.

The sebaceous glands produced in sheep skin explants were much larger than those in explants of mouse skin, and were nearer to the normal in size and extent of differentiation. They developed *in vitro* from follicles initially at stage *F*1. This seems to be the best differentiation of sebaceous glands obtained *in vitro* for any species, since other tissue cultures of sebaceous glands (Zymbal 1933) have not shown any growth of glands or differentiation of gland cells.

There are no sudoriferous glands on the general body surface of the mouse, and the present work includes what is apparently the first report of the origin and differentiation of those structures *in vitro* for any species, since other authors have obtained only undifferentiated epithelial growth (e.g. Pinkus 1938). Sudoriferous glands formed *in vitro*, elongated, and formed a small distal lumen, although development was slower than normal. Glands already present at explantation differentiated at a normal rate and developed a large distal lumen.

(c) *Development of Follicle Groups* in vitro

It was concluded that the capacity of sheep skin *in vitro* for building up a follicle group by the addition of new types of follicles in a particular arrangement was more limited than its capacity for the development of individual follicles. This may or may not be related to the failure of the skin surface area to increase at a normal rate *in vitro*. The abnormally rapid rate of development of *PCX* follicles, together with the failure of initiation of *S* follicles, in skin from the 80-day foetus, is of particular interest. This process in an intact foetus would probably result in a fleece not unlike that of primitive or carpet-wool breeds, in which the *P* fibres are very large and the *S* fibres are very much smaller and relatively few in number (Carter 1955). It is not known whether these breeds show such a delay in the initiation of *S* follicles or rapid rate of progress of *P* follicles; Ross (1954) compared the follicle group development in normal Romneys and *N*-type Romneys which produce a carpet-wool type of fleece and found no differences up to 88 days foetal age, but after this, development in *N*-type appeared to be more rapid: "*N*-type is judged to be slightly ahead, by 2 days, in the 92-, 96-, and 104-day samples . . ." Thus it is possible, but by no means certain, that more precise observations would show a more rapid rate of *P* follicle development and possibly a delay in *S* follicle initiation at this period in carpet-wool types of sheep. If so, Dry's (1934) "prenatal check" or the theory of competition for available nutrients (Fraser 1951) or both of these might be demonstrated in Merino skin *in vitro* by choosing different conditions in which *S* follicle initiation was normal, delayed, and inhibited respectively.

(d) *Potential Applications*

Among the many problems of follicle development which could be studied by tissue culture, it seems that the following might be profitable: the effect of skin expansion on follicle initiation and group formation (provided that a suitable means can be devised for mechanically stretching the skin); the effect of various conditions of cultivation on the origin of *SO* follicles and the extent of their branching to form *SD* follicles; and the effect of the retardation of follicles of one type on the development of those of other types.

The effect of temperature on wool follicle and fibre development could be tested simply *in vitro*. With a basic medium of plasma and embryo extract, it would also be possible to test the effect of adding to the skin substances such as glucose, cystine, protein hydrolysates, or copper, thus helping to separate direct from indirect effects on wool growth. The development of synthetic media suitable for differentiated growth, which is expected in the near future, will enable these and other nutritional experiments to be carried out in a more satisfactory manner. The present technique would also be adequate for examining the direct effects of vitamins or hormones on the skin and wool follicles; other keratinizing epidermal structures have recently been studied in this way (Fell and Mellanby 1953; Hardy, Biggers, and Claringbold 1953). It should be emphasized, however, that these methods are unsuitable for highly quantitative work or for examining factors which produce only small differences in the rate of follicle development or wool production.

V. ACKNOWLEDGMENTS

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VI. REFERENCES

AUBER, L., and RYDER, M. L. (1956).—Anomalies in structure and development of wool. *Proc. Int. Wool Text. Res. Conf. Aust.* **F**: 36–62.

CARTER, H. B. (1943).—Studies in the biology of the skin and fleece of sheep. 1. The development and general histology of the follicle group in the skin of the Merino. Coun. Sci. Industr. Res. Aust. Bull. No. 164. pp. 7–21.

CARTER, H. B. (1955).—The hair follicle group in sheep. *Anim. Breed. Abstr.* **23**: 101–16.

CARTER, H. B., and HARDY, MARGARET H. (1947).—Studies in the biology of the skin and fleece of sheep. 4. The hair follicle group and its topographical variations in the skin of the Merino foetus. Coun. Sci. Industr. Res. Aust. Bull. No. 215.

DAVIDSON, PAMELA, and HARDY, MARGARET H. (1952).—The development of mouse vibrissae *in vivo* and *in vitro*. *J. Anat., Lond.* **86**: 342–56.

DRY, F. W. (1934).—Hairy fibres of the Romney sheep. IV. Fibre type arrays and hairiness. *N.Z. J. Agric.* **48**: 331–43.

FELL, HONOR B. (1940).—The application of tissue culture *in vitro* to embryology. *J. R. Micr. Soc.* **60**: 95–112.

FELL, HONOR B. (1951).—Histogenesis in tissue culture. In "Cytology and Cell Physiology." 2nd Ed. pp. 419–43. (Ed. G. H. Bourne.) (Clarendon Press: Oxford.)

FELL, HONOR B., and MELLANBY, E. (1953).—Metaplasia produced in cultures of chick ectoderm by high vitamin A. *J. Physiol.* **119**: 470–88.

FRASER, A. S. (1951).—Competition between skin follicles in sheep. *Nature* **167**: 202–3.

HARDY, MARGARET H. (1949).—The development of mouse hair *in vitro* with some observations on pigmentation. *J. Anat., Lond.* **83**: 364–84.

HARDY, MARGARET H. (1951).—The development of pelage hairs and vibrissae from skin in tissue culture. *Ann. N.Y. Acad. Sci.* **53**: 546–61.

HARDY, MARGARET H., BIGGERS, J. D., and CLARINGBOLD, P. J. (1953).—Vaginal cornification of the mouse produced by oestrogens *in vitro*. *Nature* **172**: 1196–7.

HARDY, MARGARET H., and LYNE, A. G. (1956*a*).—Proposed terminology for wool follicles in sheep. *Nature* **177**: 705–6.

HARDY, MARGARET H., and LYNE, A. G. (1956*b*).—The histological development of the skin and wool in the Merino foetus. *Proc. Int. Wool Text. Res. Conf. Aust.* **F**: 26–31.

HARDY, MARGARET H., and LYNE, A. G. (1956*c*).—The development of wool follicles from foetal sheep skin in tissue culture. *Proc. Int. Wool Text. Res. Conf. Aust.* **F**: 32–5.

HARDY, MARGARET H., and LYNE, A. G. (1956*d*).—The pre-natal development of wool follicles in Merino sheep. *Aust. J. Biol. Sci.* **9**: 423–41.

MURRAY, MARGARET R. (1933).—Development of the hair follicle and hair *in vitro*. *Anat. Rec.* **57** (4) (suppl.): 74.

PINKUS, H. (1938).—Notes on structure and biological properties of human epidermis and sweat gland cells in tissue culture and in the organism. *Arch. exp. Zellforsch.* **22**: 47–52.

PINKUS, H. (1951).—Multiple hairs (Flemming-Giovannini). Report of two cases of Pili multigemini and discussion of some other anomalies of the pilary complex. *J. Invest. Derm.* **17**: 291–301.

ROSS, JANET M. (1954).—Development of the follicle population in *N*-type sheep. *Aust. J. Agric. Res.* **5**: 484–9.

Strangeways, D. H. (1931).—The growth of hair in vitro. *Arch. exp. Zellforsch.* **11**: 344–5.

Thomas, J. A., Salomon, L., Salomon, Léone, and Lamy, F. (1948).—La survie expérimentale aseptique des grands foetus de Mammifères. *C. R. Acad. Sci., Paris* **226**: 966–8.

Zymbal, W. E. (1933).—Histologische und experimentelle Untersuchungen am Epithelgewebe der Talgdrüsen (Gehörgangdrüse der Ratte). *Z. Zellforsch.* **18**: 596–625.

Explanation of Plates 1–3

All figures are of sections through follicles or associated glands or both in skin of the midside from sheep foetuses directly or after cultivation. The staining is with haemalum, eosin, and picric acid. Sections shown in Plate 2, Figure 2, and Plate 3, Figure 1 were fixed in formalin, and all others in Zenker's fluid. *B*, basement membrane; *Cu*, cuticle of wool fibre; *D*, dermis; *DP*, dermal papilla of wool follicle; *G*, stratum germinativum; *H*, keratinized wool fibre; *He*, Henle's layer; *Hu*, Huxley's layer; *HN*, hair cone; *I*, inner root sheath; *ICu*, inner root sheath cuticle; *L*, lumen of sudoriferous gland; *O*, outer root sheath; *P*, periderm; *PP*, pre-papilla; *S*, stratum spinosum; *SG*, sebaceous gland with differentiated cells; *SudG*, sudoriferous gland; *SudGD*, sudoriferous gland duct; *SudGR*, sudoriferous gland rudiment.

Plate 1

Development of wool follicles in tissue culture

Fig. 1.—Vertical section of skin from 70-day foetus with a *PCX* follicle at stage *F*1.

Fig. 2.—Horizontal section of skin from 70-day foetus with a *PCX* follicle at stage *F*1.

Fig. 3.—Longitudinal section of *PCX* follicle at stage *F*2 in explant of skin from 70-day foetus at 74 days.

Fig. 4.—Longitudinal section of *P* follicle at stage *F*3*b* showing sudoriferous gland rudiment in explant of skin from 70-day foetus at 101 days.

Fig. 5.—Longitudinal section of *P* follicle at stage *F*4 in explant of skin from 70-day foetus at 88 days.

Fig. 6—Longitudinal section of *P* follicle at stage *F*7 in explant of skin from 70-day foetus at 108 days.

Plate 2

Development of wool follicles in tissue culture

Fig. 1.—Oblique section of *P* follicle at stage *F*7 at level of sebaceous gland in explant of skin from 70-day foetus at 101 days.

Fig. 2.—Transverse section of *PC* follicle at stage *F*7 at level of sebaceous gland in skin from from 100-day Merino foetus.

Fig. 3.—Transverse section of sudoriferous gland associated with the *P* follicle shown in Plate 2, Figures 1 and 5.

Fig. 4.—Section of sebaceous gland showing secretory process in explant of skin from 70-day foetus at 108 days.

Fig. 5.—Longitudinal section of base of *P* follicle shown in Plate 2, Figure 1.

Fig. 6.—Longitudinal section of base of *PC* follicle at stage *F*5 in skin from 98-day Merino foetus.

Plate 3

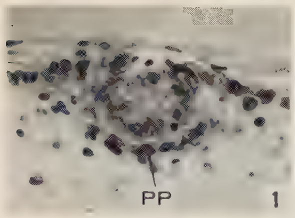
Development of wool follicles in tissue culture

Fig. 1.—Vertical section of skin from 80-day foetus with a *PCX* follicle at stage *F*2*a*.

Fig. 2.—Part of explant of skin from 80-day foetus at 84 days, showing follicle on left at stage *F*3*a* and follicle on right at stage *F*1.

Fig. 3.—Horizontal section of entire explant of skin from 80-day foetus at 84 days, showing development of follicles around central epidermis.

WOOL FOLLICLE DEVELOPMENT IN TISSUE CULTURE



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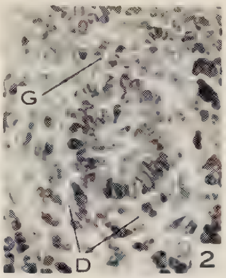

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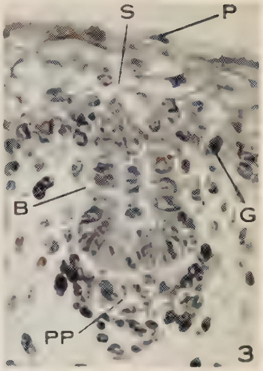

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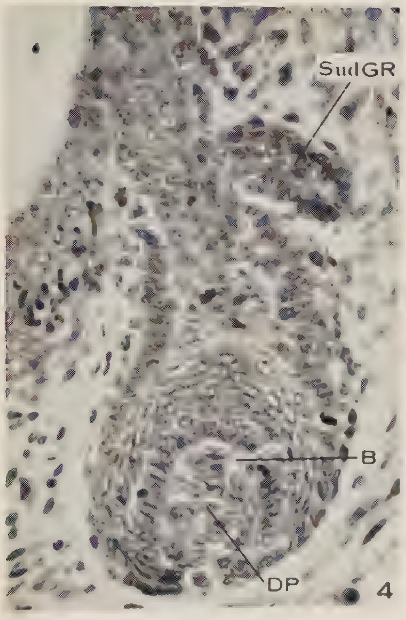

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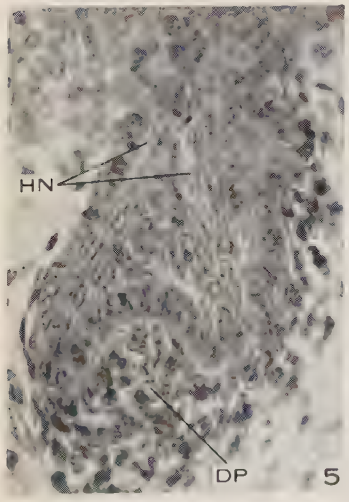

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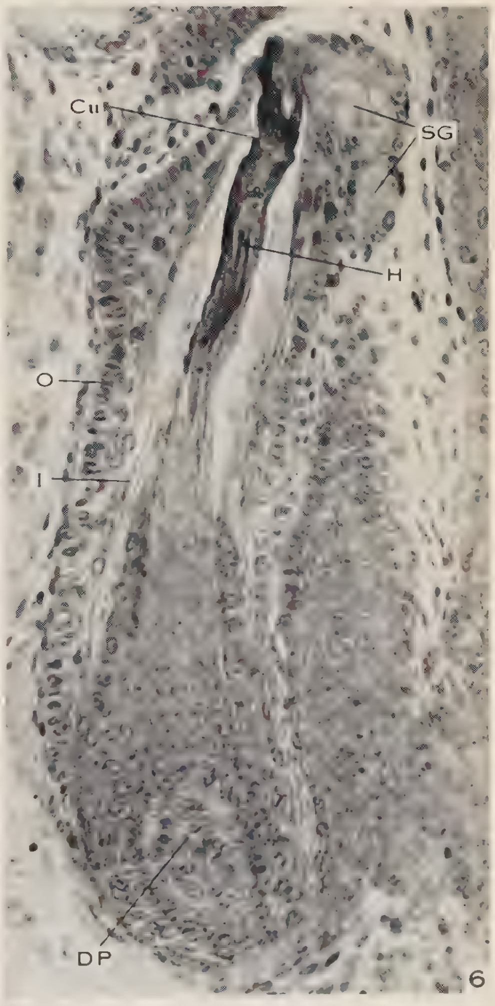

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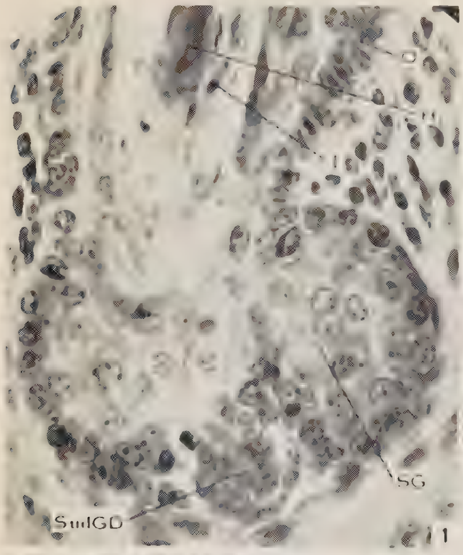
WOOL FOLLICLE DEVELOPMENT IN TISSUE CULTURE



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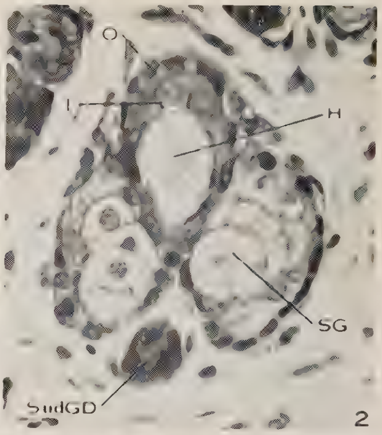

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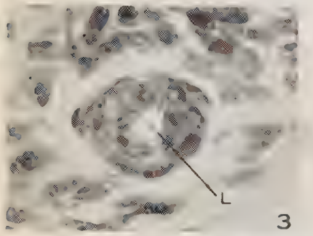

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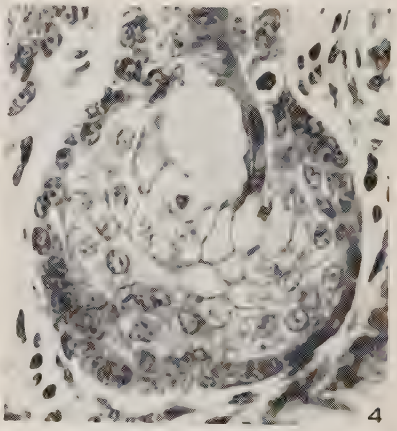

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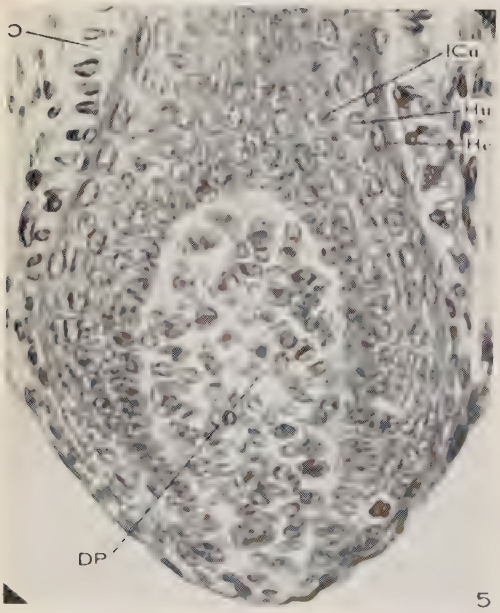

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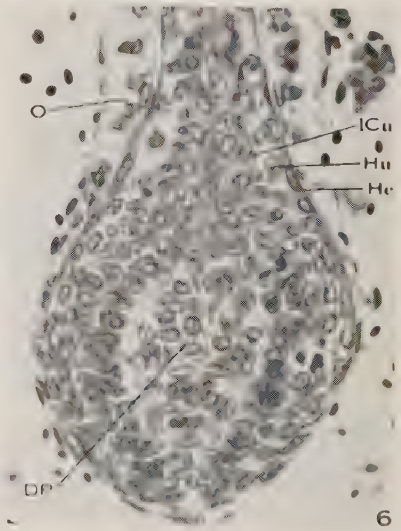

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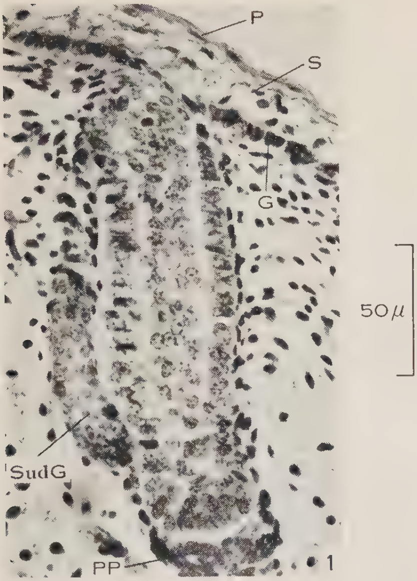

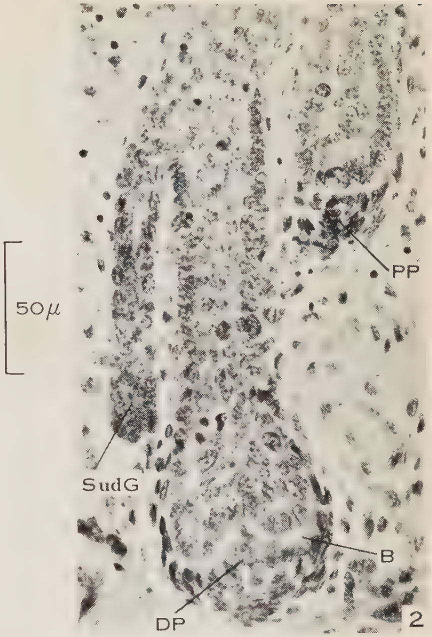

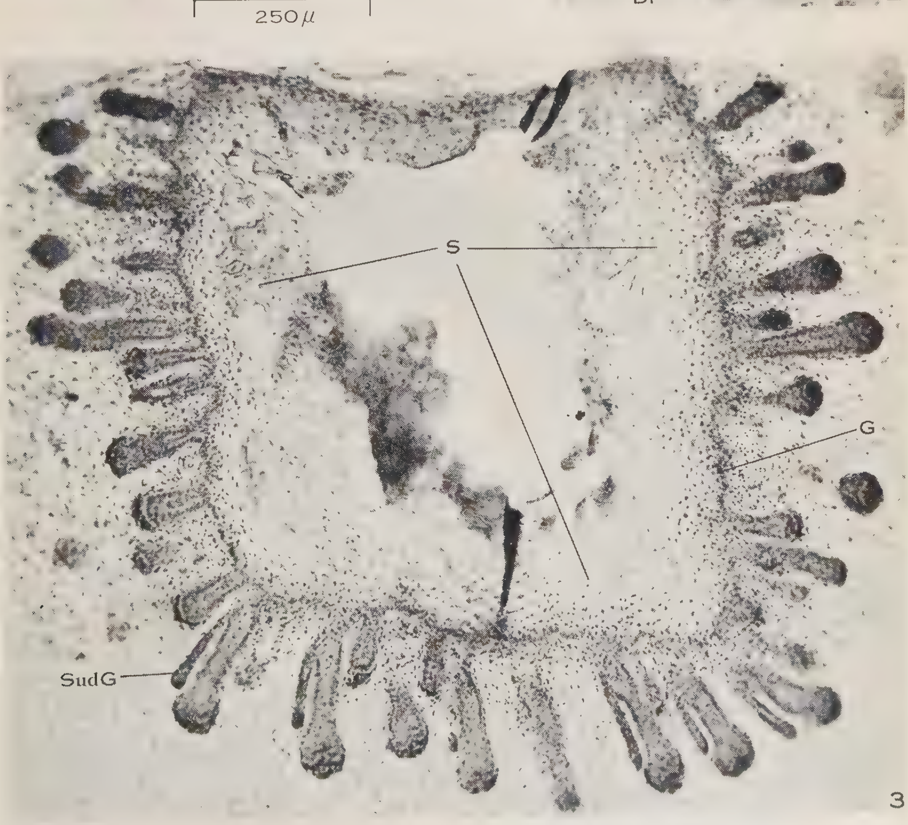

THE INFLUENCE OF THE THYROID ON WOOL FOLLICLE DEVELOPMENT IN THE LAMB*

By K. A. Ferguson,† P. G. Schinckel,† H. B. Carter,‡ and W. H. Clarke†

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Summary

Thyroidectomy of the newborn lamb prevents the maturation of secondary wool follicles. The administration of L-thyroxine to thyroidectomized lambs allows normal follicle development.

Body growth and wool growth are also depressed by thyroidectomy and most thyroidectomized lambs do not survive more than a few weeks without replacement therapy. The effects on wool growth do not appear to be secondary to the effects on follicle development.

The thyroxine requirements for normal wool growth and for normal secondary follicle maturation appear to be greater than the requirements for general body growth.

I. Introduction

The development of wool follicles in the lamb has been described by Carter (1943), Carter and Hardy (1947), Burns (1949, 1954), Fraser (1954), Schinckel (1953, 1955), and Short (1955). Primary follicles are fully developed and are producing fibres at birth. Development of most if not all secondary follicles commences by the time of birth but in the Merino only about 20 per cent. of secondary follicles are producing fibres at this time. In the first month after birth this proportion increases to 70–80 per cent. Further development then occurs more slowly.

The thyroid is well known to affect growth and differentiation. Simpson (1924) found that thyroidectomy of lambs retarded growth and reduced fleece weights at 13–14 months of age by 50 per cent. but no observations were reported on follicle development. The hair cycles of rats are greatly retarded by thyroidectomy (Salmon 1938; Scow and Marx 1945; Scow and Simpson 1945; Dicke 1948), and the administration of throxine to normal rats has been reported to accelerate the hair cycle (Butcher 1937). The lamb does not exhibit several cycles of wool development similar to the hair cycles in the rat, but development of individual follicles appears to be a similar process in both species.

This paper reports the results of an experiment carried out to determine the effect of the thyroid on the maturation of the secondary follicles of the lamb. The results show that the thyroid does markedly influence this process.

II. Materials and Methods

Twenty-six pregnant 6-year-old ewes of the "Egelabra" medium wool Merino strain were housed in individual indoor pens and fed *ad libitum* on a ration of 50 parts by weight of lucerne chaff, 20 parts of wheat, 10 parts of crushed oats, 10 parts of linseed meal, and 10 parts of coco-nut meal.

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* A brief account of this work was presented to Section L, A.N.Z.A.A.S., January 1954.

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The lambs were allotted at random to the following four groups:

Group A.—The six lambs in this group were untreated controls.

Group B.—Eight lambs were thyroidectomized during the first week of life. Thyroxine injections into the four surviving lambs were commenced after 11 weeks of age by which time secondary follicle development in the control group had passed its most active phase. Pure sodium-L-thyroxine was injected in a dosage of 40 mg per unit of weight (expressed as $kg^{0 \cdot 73}$) per day. This was estimated to be a little more than the normal secretion rate based on the data of Schultze and Turner (1945) for young goats. The thyroxine was dissolved in 0·9 per cent. NaCl at pH 9·3 and injections given subcutaneously. Dosages were adjusted to body weight weekly.

Group C.—Six lambs were thyroidectomized during the first week of life. Thyroxine injections were given from the day of operation until 11 weeks of age. The dose rate was the same as for group B.

Group D.—Four lambs were injected daily with a dilute $Ca(OH)_2$ (pH 11·5) extract of sheep pituitary glands. The extract was freeze-dried, and, for injection, dissolved in 0·9 per cent. NaCl at pH 9·0. Solution was not complete at this pH but the fine suspended matter was not removed prior to injection. The lambs received 10 mg dry weight of extract per unit body weight in $kg^{0 \cdot 73}$. The extract was assayed for thyrotrophic hormone (thyroid weight increase in chicks (Smelser 1937, 1938)) and growth hormone (body weight increase in hypophysectomized rats (Evans and Simpson 1931; Marx, Simpson, and Evans 1942)). 10 mg of the dry extract was found to contain 0·5 mg of thyrotrophic hormone activity in terms of the Armour preparation 129–31 PRR3, and 1 mg of growth hormone activity in terms of a standard of purified ox growth hormone, prepared by the method of Wilhelmi, Fishman, and Russell (1948).

The lambs were weighed and the skin sampled at birth and thereafter on Wednesday of each week except in lambs then 2 days old or younger, in which case the second sample was taken on the following Wednesday. The skin samples were taken by means of a trephine which cut a circle of skin 1 cm in diameter and were fixed in 5 per cent. formol saline. Successive samples were taken from alternate midsides, the sample on each side being taken about 1 cm behind the previous sample until the 12th sample when another row below the first was commenced in the reverse direction. After the 17th sample the interval between sampling was increased to 2 weeks, and after the 19th sample to 4 weeks so that the 22nd sample was taken at 32 weeks of age. One further sample was taken at 56 weeks of age when the experiment was completed.

Histological sections 7–8 μ thick were cut horizontal to the surface of the skin and stained with haemalum, picric acid, and eosin. Counts of primary (P) and secondary (S) fibres were made at a linear magnification of ×115 counting six to 12 fields depending on the number of fibres present. The counts were corrected for the shrinkage which occurred during fixation and processing by measuring the area of the section.

Wool samples were clipped from areas defined by tattoo lines on the left midside region. The lines were tattooed on the day of the second skin sampling, i.e. 3–9 days after birth; the sample areas were initially 10 sq. cm, subsequently increasing in size as the lambs grew. The wool samples were taken after consecutive periods of 4, 4, 2, 4, 5, and 4 weeks except in a few cases when the proper sampling days were missed. The samples were extracted with ether and cold water and were oven dried at 100°C prior to weighing.

Estimates of milk consumption of the lambs were obtained by test feeding at weekly intervals until 15 weeks of age. Milk consumption was recorded by weighing the lambs before and after suckling at the end of three 3-hourly periods on the day of test. In addition to milk the lambs had free access to the food of the ewes. The lambs were weaned at 18 weeks of age.

III. Results

(a) *Completeness of Thyroidectomy*

Variation in the effects produced by thyroidectomy indicated the presence of accessory thyroid tissue in some lambs or incomplete removal of the thyroid at the time of operation.

Table 1

WET WEIGHTS OF THYROID FRAGMENTS FOUND AT POST-MORTEM IN THYROIDECTOMIZED SHEEP*

Group	Lamb No.	Thyroid Fragment Weight (g)
B	A24	0·15
	A56	0·05
	A58	0·03
C	A32	0·01

* The weight of the combined thyroid glands of normal sheep of the same age is 4–5 g.

Autopsy at the conclusion of the experiment showed the presence of hypertrophied thyroid fragments in three lambs in group B and in one lamb in group C in the region of the thyroid isthmus. The weights of the fragments are shown in Table 1. The nature of the tissue was confirmed by histological examination. No thyroid fragments could be found in any of the other thyroidectomized lambs.

(b) *General Effects of Thyroidectomy*

After 2 weeks the thyroidectomized lambs in group B were noticeably less active, becoming dull, stunted, and pot-bellied. They had a typical cretinoid appearance. Muscular weakness and inability to stand was noted especially in the morning after a cool night. Four lambs in this group died and if thyroxine treatment had not been initiated after 11 weeks of age two more would probably have died. Most of the lambs in this group were constipated in the period prior to receiving thyroxine injections. The four deaths were due to the following immediate causes: peritonitis from perforation of the colon by hard faecal material; pneumonia; weakness and inanition; and laryngeal paralysis presumably resulting from interference with the recurrent laryngeal nerve at the time of operation. Total thyroidectomy of the newborn lamb must be considered a fatal operation since, of the four lambs surviving until thyroxine injections were given, three possessed fragments of thyroid tissue.

In group C signs of thyroid deficiency began to appear about 5 weeks after the cessation of thyroxine injections. The symptoms were similar to those appearing in the younger lambs in group B.

(c) *Milk Consumption*

Milk consumption was depressed in group B relative to the other groups which were not significantly different from one another.

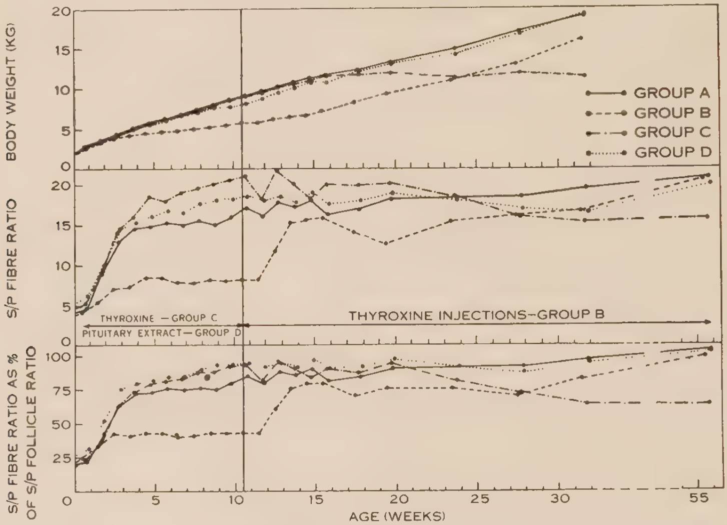

Fig. 1.—Mean body weight, *S/P* fibre ratio, and *S/P* fibre ratio as percentage of *S/P* follicle ratio for each group.

(d) *Growth*

The mean body weight of each group is shown in Figure 1. In group B growth virtually ceased 2 weeks after thyroidectomy except in two lambs (A24 and A58) which grew at nearly normal rate. It may be seen from Table 1 that these two lambs had thyroid fragments at post-mortem. Growth recommenced in the other two surviving lambs of this group 4 weeks after thyroxine injections were started at 11 weeks of age and the growth curve of this group became parallel to that of the control group.

The growth of lambs in group C was similar to that of the controls during the period they received thyroxine injections. Five weeks after the cessation of injections further body growth ceased and body weight remained level until the conclusion of the experiment. The milk supply of one ewe in group C was poor and was supplemented by bottle feeding. However, the growth rate of the lamb A32 was well below normal and the mean body weight curve for this group in Figure 1 excludes the data for this lamb.

The lambs in group D receiving pituitary extract grew at a similar rate to that of the controls during the period of injections. It may be noted that the growth of groups C and D was not increased above normal by the administration of thyroxine and pituitary extract respectively during the first 11 weeks of age.

(*e*) *Secondary Wool Follicle Maturation*

The ratio of secondary to primary (S/P) fibres appears to be the most suitable measure of the maturation of secondary follicles in the lamb (Carter 1943; Schinckel 1955). The validity of the measure requires that primary follicle development be substantially complete by the time of birth.

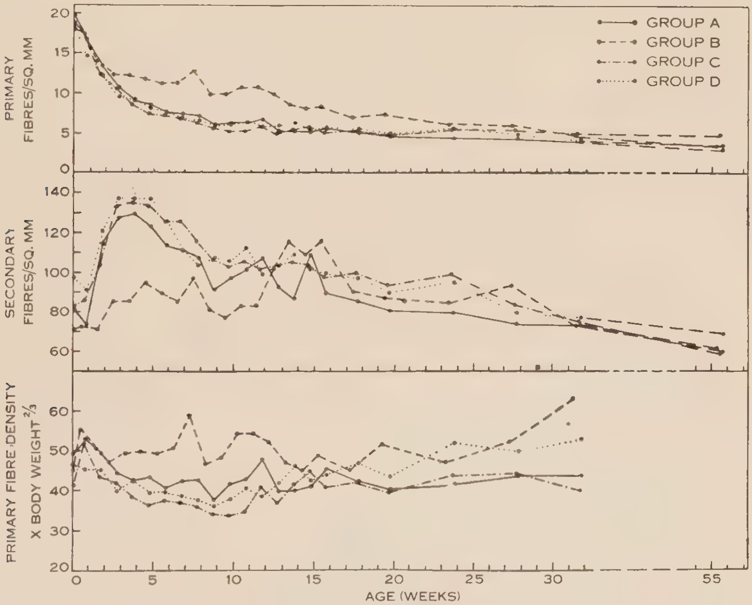

Fig. 2.—Mean primary fibre population density, secondary fibre population density, and primary fibre population density × body weight in kg$^{\frac{2}{3}}$ for each group.

No histological evidence of further primary follicle development after birth was found in the present experiment and estimates of the total primary fibre population given by the product of body weight to the $\frac{2}{3}$ power and primary fibre population density for each group show no consistent increase with age (Fig. 2). The greater values of the product for group B during the first 11 weeks of age are probably due to the increased amount of skin taken in the biopsy sample caused by the huddled attitude of these lambs. A similar increase in primary density is also likely to be the explanation of the increased values of the product for all groups for the early samples.

Figure 1 shows the S/P fibre ratio and this ratio expressed as a percentage of the S/P follicle ratio at birth. The individual data for these percentages are shown in Figure 3. The expression of the data in this form removes variability resulting from differences in the potential mature *S*/*P* fibre ratio but, on the other hand, introduces variability due to the sampling error of the measurement of *S*/*P* follicle ratio at birth.

After thyroidectomy there was little further secondary follicle maturation in the lambs of group B except in one lamb (A24) which showed normal body growth and which had the largest thyroid fragment remaining at the conclusion of the experiment. However, follicle maturation showed a sudden increase in this lamb after the start of thyroxine injections at 11 weeks of age. The other lamb in group B with normal body growth (A58) showed a more marked depression of secondary follicle development. This lamb also responded immediately to thyroxine injections. The other two lambs in group B which survived the experiment showed a much slower follicle maturation response to thyroxine but eventually maturation was complete at 12 months of age.

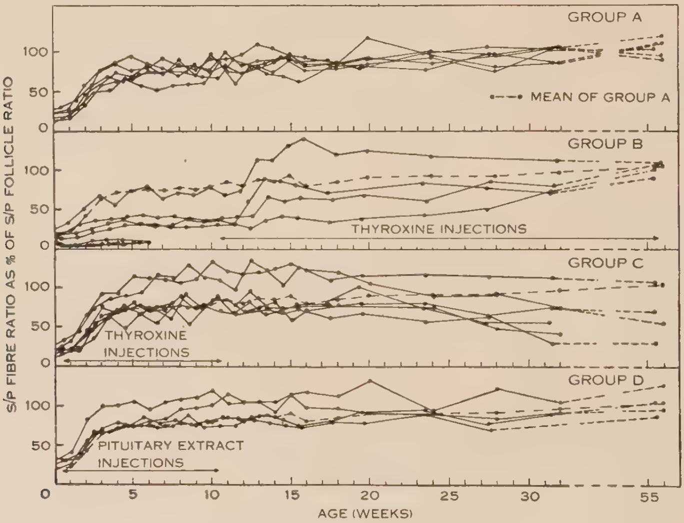

Fig. 3.—Individual values of *S*/*P* fibre ratio at various ages as a percentage of *S*/*P* follicle ratio for each group.

The depression of secondary follicle maturation may be seen in Figure 3 to have been much more marked in the four lambs which died than in the surviving lambs of group B in which thyroid deficiency may have been less severe. However, except in one instance, the body growth of the four lambs which died was similar to that of the survivors. Apparently a level of thyroxine secretion sufficient for general body growth is not sufficient for the normal rate of secondary follicle maturation. The results also indicate that the effect of thyroidectomy on secondary follicle maturation is not a secondary effect of diminished growth and food intake. This conclusion may be examined by plotting the *S*/*P* fibre ratio against body weight to the $\frac{2}{3}$ power from birth until thyroxine injections were commenced in group B (Fig. 4). The individual data are shown for the control group separately with each of the treated groups. It may be seen that at comparable body weights follicle maturation was less in the thyroidectomized lambs of group B.

The secondary fibre population density of the lambs in group B (see Fig. 2) was far below that of the other groups during the active stage of secondary follicle development. This supports the conclusion from Figure 4 that a crowding effect due to lack of skin growth was not the cause of the depression of secondary follicle maturation. Figure 2 also shows that the increase in follicle development in the first few weeks of life in normal lambs is not due to increased "living" space for follicles. Rather the development proceeds in spite of increasing fibre population density. The primary fibre density was of course greater in the lambs of group B due to their diminished growth.

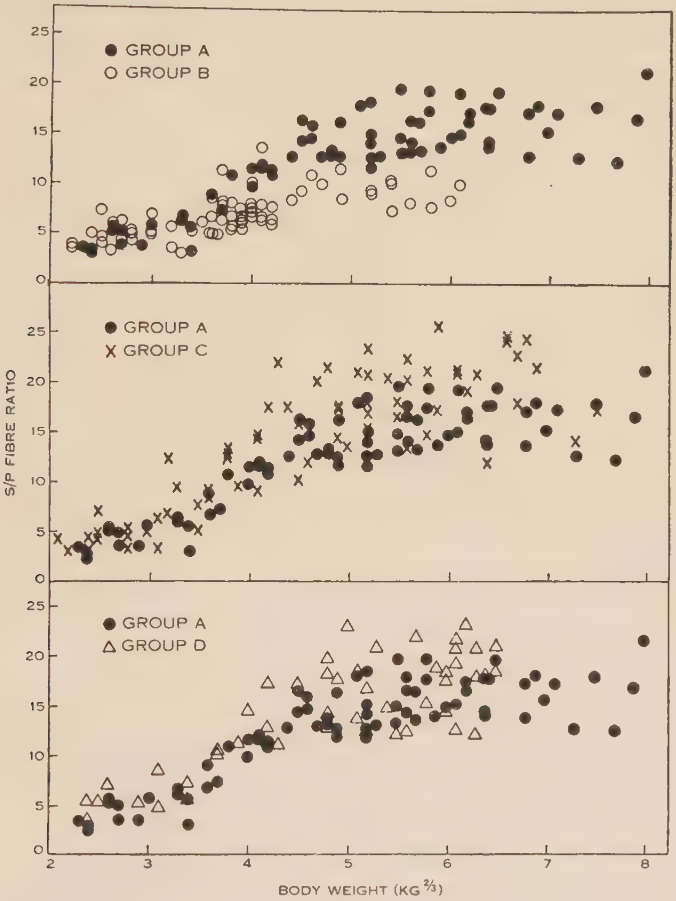

Fig. 4.—Relation of S/P fibre ratio to body weight in $\text{kg}^{2/3}$. Group A is compared separately with groups B, C, and D.

The thyroidectomized lambs of group C which received thyroxine during the period of active follicle development showed an apparently more rapid secondary follicle maturation than the control lambs. However, the mean S/P fibre ratio, expressed as a percentage of the S/P follicle ratio (Fig. 1), during the period of injections for group C was not significantly greater than for the controls. After injections ceased there was a gradual decline in the S/P fibre ratio owing to the atrophy of some secondary follicles. Some primary follicles also shed their fibres but to a lesser degree than did the secondaries.

One lamb in group C which grew at a subnormal rate due to the failure of its dam's milk supply showed a depressed secondary follicle maturation but maturation was greater than in the lambs of group B despite a similar body growth.

The lambs of group D which received injections of sheep pituitary extract during the period of active secondary follicle maturation also showed an apparently more rapid development than the control lambs but again the difference was not statistically significant.

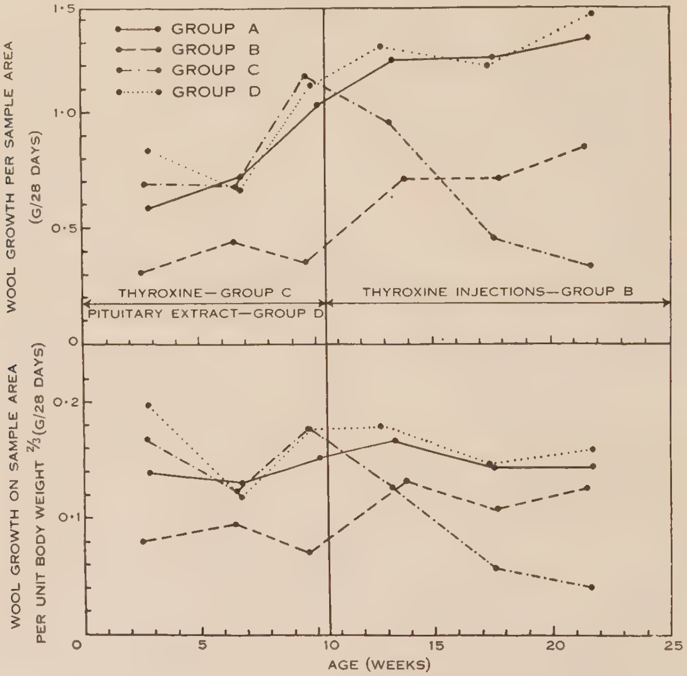

Fig. 5.—Mean wool growth per sample area and wool growth on sample area per unit body weight in $\text{kg}^{\frac{2}{3}}$ for each group.

(*f*) *Wool Growth Rate*

When the weight of wool grown per unit time on the tattooed sample areas is used as a measure of the total wool growth rate of the lamb, it is subject to the error introduced by variation in size of the lambs when they were tattooed. This error can be corrected by multiplying the individual wool weights per sample area throughout the experiment by a factor for each lamb. This factor is given by $W^{\frac{2}{3}}$(individual)/$W^{\frac{2}{3}}$(mean of all lambs), where W equals body weight at the time of tattooing.

The wool growth rates per sample area for each group adjusted in this way are shown in the upper half of Figure 5. The regression coefficient for the regression of log wool growth per sample area on log body weight for the period means of groups

A and D (which did not differ significantly from one another) was found to be $0{\cdot}676 \pm 0{\cdot}200$. This indicates that the sample area wool growth per unit body weight to the $\frac{2}{3}$ power was independent of body weight in these two groups from birth until 6 months of age when wool growth measurements were terminated. Figure 5 also shows the wool growth rates on the sample area per unit body weight in kg$^{\frac{2}{3}}$. The relation of wool growth per sample area to body weight in kg$^{\frac{2}{3}}$ for groups A and D is shown in Figure 6.

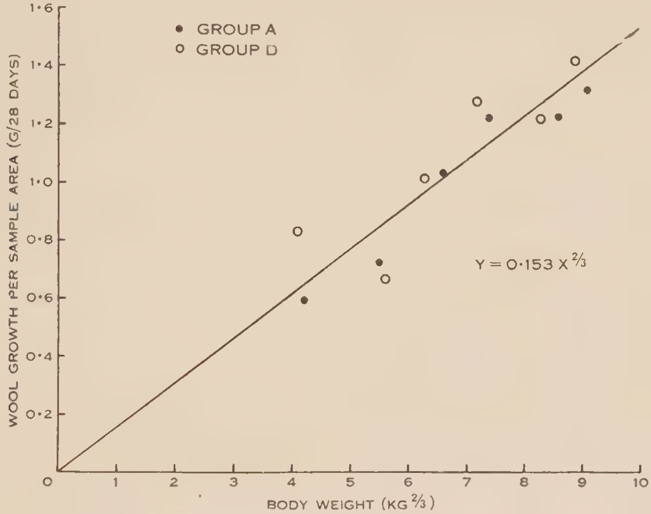

Fig. 6.—Relation of wool growth per sample area to body weight in kg$^{\frac{2}{3}}$ for groups A and D.

In group B wool growth per sample area was considerably depressed but to a lesser extent in the two lambs in this group which showed normal body growth. Thyroxine caused an increase in wool growth but it remained substantially less than in the control lambs up till 6 months of age. Wool growth per unit body weight in kg$^{\frac{2}{3}}$ was also depressed during the pre-injection period and did not reach normal after thyroxine injections were given. Thus the depression of wool growth per sample area cannot be attributed to the smaller size of these lambs. The extent to which wool growth per unit body weight in kg$^{\frac{2}{3}}$ of different lambs approached the control level when thyroxine was given was not related to the corresponding S/P fibre ratio or to fibre population density. Furthermore, in any wool growth period there was no relation between wool growth per unit body weight in kg$^{\frac{2}{3}}$ and S/P fibre ratio or fibre population density within any of the groups.

In group C wool growth per sample area and per unit body weight in kg$^{\frac{2}{3}}$ was similar to that of the control lambs while they received thyroxine. When thyroxine injections ceased, wool growth, expressed in both ways, declined sharply.

IV. Discussion

The present experiment shows that the thyroid is necessary for the completion of secondary follicle development in the lamb as indeed it is for growth and development in general. However, it appears that the requirement of the follicles for thyroxine is greater than that of the body for growth in general. Natural conditions might exist under which thyroxine secretion might be inadequate for normal secondary follicle maturation.

In the present experiment depression of secondary follicle maturation for 11 weeks by thyroid deficiency did not lead to permanent depression of the S/P fibre ratio. However, it did cause an effect which took months of thyroxine injections to repair and it is possible that a more prolonged period of thyroid deficiency could lead to a permanent loss of secondary follicles.

Current experiments at this Laboratory (Schinckel and Short, unpublished data) show that restricted post-natal nutrition delays the maturation of secondary follicles, but it is too early to state whether there is a permanent depression of secondary follicle development.

Poor nutrition leads to depression of secretion of thyrotrophic hormone and hence of thyroxine in the guinea pig (Stephens 1940) and this effect, if present in the sheep, may account at least in part for the delayed follicle development of lambs fed on a restricted diet.

Schinckel (1953) has shown that the lambs from twin births have a retarded maturation of secondary follicles compared with single lambs. Not only is maturation retarded but also the S/P fibre ratio of twins appears to remain permanently below that of singles. However, it is not clear to what extent the difference between singles and twins results from post-natal nutrition.

Depression of wool growth rate accompanied the depression of follicle maturation caused by thyroid deficiency but the effect on wool growth did not appear to be a consequence of the lowered follicle population but rather to be independent of it. This suggests that the two effects may be referable to different metabolic defects which occur to different relative extents in different lambs.

V. Acknowledgments

We are indebted to Mr. W. T. Outch, Mr. W. H. Blair, Miss C. Bathgate, Miss J. Bathgate, and Miss H. Bloomfield for technical assistance.

Our thanks are due to Glaxo Laboratories for the gift of thyroxine and to Mr. E. K. Bowman of "Wargundy", Craboon, N.S.W., who generously donated the sheep used in this investigation.

VI. References

Burns, M. (1949).—*J. Agric. Sci.* **39**: 64.
Burns, M. (1954).—*J. Agric. Sci.* **44**: 86.
Butcher, E. O. (1937).—*Amer. J. Physiol.* **120**: 427.
Carter, H. B. (1943).—Coun. Sci. Industr. Res. Aust. Bull. No. 164.
Carter, H. B., and Hardy, M. H. (1947).—Coun. Sci. Industr. Res. Aust. Bull. No. 215.
Dicke, S. H. (1948).—*Endocrinology* **42**: 315.
Evans, H. M., and Simpson, M. E. (1931).—*Amer. J. Physiol.* **98**: 511.
Fraser, A. S. (1954).—*Aust. J. Agric. Res.* **5**: 737.
Marx, W., Simpson, M. E., and Evans, H. M. (1942).—*Endocrinology* **30**: 1.
Salmon, T. N. (1938).—*Endocrinology* **23**: 446.

Schinckel, P. G. (1953).—*Nature* **171**: 310.
Schinckel, P. G. (1955).—*Aust. J. Agric. Res.* **6**: 68.
Schultze, A. B., and Turner, C. W. (1945).—Res. Bull. Mo. Agr. Exp. Sta. No. 392.
Scow, R. O., and Marx, W. (1945).—*Anat. Rec.* **91**: 227.
Scow, R. O., and Simpson, M. E. (1945).—*Anat. Rec.* **91**: 209.
Short, B. F. (1955).—*Aust. J. Agric. Res.* **6**: 62.
Simpson, S. (1924).—*Quart. J. Exp. Physiol.* **14**: 185.
Smelser, G. K. (1937).—*Proc. Soc. Exp. Biol., N.Y.* **37**: 388.
Smelser, G. K. (1938).—*Endocrinology* **23**: 429.
Stephens, D. J. (1940).—*Endocrinology* **26**: 485.
Wilhelmi, A. E., Fishman, J. B., and Russell, J. A. (1948).—*J. Biol. Chem.* **176**: 735.

THE IODINE COMPOUNDS OF THYROID AND PLASMA STUDIED BY COLUMN CHROMATOGRAPHY

By T. H. Kennedy* and H. D. Purves*

[*Manuscript received June 8, 1956*]

Summary

A system of column chromatography suitable for the separation of the biologically important iodine compounds is described. Analysis of hydrolysed thyroid tissue shows the presence of thyroxine, 3,5,3′-tri-iodothyronine (TIT), 3,5-di-iodotyrosine (DIT), mono-iodotyrosine (MIT), and other unidentified iodine-containing compounds. The relative abundance of thyroxine and TIT present as free amino acids in the thyroid is different from that of the same amino acids in protein combination. The amount of free thyroxine is less than the free TIT, whereas in the combined amino acids thyroxine is in excess of TIT.

TIT was not found in the plasma of the rat except when radiation damage to the thyroid gland had occurred. Under these circumstances it was accompanied by MIT and DIT and an iodine-containing protein presumed to be thyroglobulin.

I. Introduction

Recent knowledge of the iodine compounds of the thyroid has been reviewed by Roche and Michel (1954) and Gross (1954). Under normal conditions most of the iodine in the thyroid gland is in the form of thyroglobulin, which contains the iodinated amino acids thyroxine, 3,5,3′-tri-iodothyronine (TIT), 3,5-di-iodotyrosine (DIT), and (MIT). These same four compounds also occur as free amino acids in the thyroid gland presumably arising from proteolytic breakdown of thyroglobulin.

Barker (1955) has reviewed the literature on the nature of the iodine compounds of plasma. Thyroxine loosely bound to protein is the major organic iodine compound in the plasma. TIT has been reported as a plasma constituent but is not a consistent finding. Neither DIT nor MIT appear to be normal constituents of plasma according to recent investigations. Iodine compounds not normally present in plasma may be released from the thyroid when this structure has suffered radiation damage from excessive doses of ^{131}I.

An iodine-containing protein was found in the plasma of rats after destructive doses of ^{131}I by Tong, Taurog, and Chaikoff (1952), and similar material appearing in human plasma was shown to be thyroglobulin by Robbins, Peterman, and Rall (1954) using physicochemical methods. Benua and Dobyns (1955) found DIT and MIT in the plasma of patients receiving large doses of ^{131}I and ascribed their presence to radiation damage.

The appearance of thyroglobulin in plasma after radiation damage to the thyroid makes it likely that any thyroid constituent could appear in plasma under the same circumstances. We have therefore investigated the nature of the iodine compounds present in thyroid and plasma and the time course of the changes induced by thyroid-damaging doses of ^{131}I in the rat. A preliminary account of this work has been published (Kennedy 1953).

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II. Materials and Methods

The iodine-containing compounds were separated by column chromatography with kieselguhr-supported sodium hydroxide solution as the stationary phase and *tert.*-butanol–*cyclo*hexane mixtures, previously equilibrated with 2N sodium hydroxide, as the mobile phase. For most separations, 5 g of kieselguhr were mixed with 3·5 ml of 2N sodium hydroxide solution and packed into a column 12 mm in diameter.

The column was started with a 70:30 (v/v) mixture of *tert.*-butanol and *cyclo*hexane and run until the thyroxine had emerged, when an 80:20 (v/v) mixture of the same solvents was applied to elute the TIT. A further change to a 90:10 (v/v) mixture eluted the iodide, after which DIT and MIT were eluted with a 90:10 (v/v) *n*-propanol–*cyclo*hexane mixture and *n*-propanol respectively. Approximately 75-ml quantities of each solvent were required.

The rats used were a Wistar strain, weight 150–200 g, and were fed with a low iodine diet. Rats maintained on this diet always have hyperplastic thyroids. This increased activity of the thyroid can be prevented by the addition of enough potassium iodide to the drinking water to supply the animals with 1–2 μg iodide daily. ^{131}I was administered in the form of iodide to produce *in vivo* labelling of the iodine compounds of the thyroid gland and hence of thyroid secretion. Preliminary tests with 5 μc doses of ^{131}I showed a 60–80 per cent. uptake in the animals used. Blood samples were taken by heart puncture and the blood centrifuged immediately. The plasma was kept frozen until required.

Plasma, 0·2–0·3 ml, was mixed with 0·1 ml of 10N sodium hydroxide, 0·5 g of kieselguhr added and made into a slurry with 3–5 ml of the mobile phase and packed on top of a column prepared as already described. The free amino acids of the thyroid were examined by grinding the thyroid with 0·2 ml of 3N NaOH, or heating the mixture in a boiling water-bath for 1 min and placing the solution on the column as for plasma.

The effluent from the column was run through an F10 liquid-flow counter (Twentieth Century Electronics) and collected in approximately 3-ml fractions. The radioactivity was measured with a rate-meter and recorded on a recording potentiometer.

Where necessary, carrier amino acids and iodide, about 1 mg of each, were added to the material to be analysed before it was placed on the column. The carrier amino acids were found in the effluent by measuring the absorption at 300 mμ. The iodide was located by addition of a few drops of a reagent made by mixing equal volumes of 6 per cent. hydrogen peroxide and 2N sulphuric acid, to which a small crystal of sodium tungstate was added. The iodine formed was estimated colorimetrically. The commercially-available grades of *tert.*-butanol, *n*-propanol, and *cyclo*hexane were suitable for absorption measurements without preliminary purification.

The radioactivity of the separated substances was measured by gamma counting after evaporation of the pooled fractions to a volume of 3–4 ml. Sufficient counts were made to ensure an accuracy of ±1·8 per cent. or less.

III. Results

The chromatographic system gave an adequate separation of a mixture of thyroxine, TIT, iodide, DIT, and MIT which emerged in that order as shown in Figure 1. 3,5-Di-iodothyronine was not completely separated from iodide by this system.

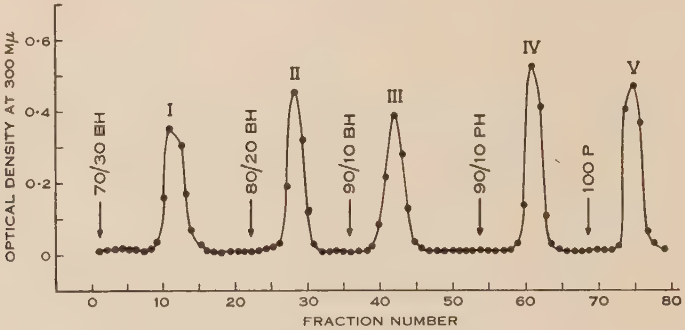

Fig. 1.—Separation of a mixture of thyroxine (I), tri-iodothyronine (II), iodide (III), di-iodotyrosine (IV), and mono-iodotyrosine (V). Solvent changes were made at the points indicated. BH, *tert.*-butanol–*cyclo*hexane (v/v) mixture; PH, *n*-propanol–*cyclo*hexane (v/v) mixture; P, *n*-propanol.

(*a*) *Total Iodo-amino Acids of the Thyroid*

When applied to the separation of the labelled iodine compounds of hydrolysed rat thyroid tissue, a satisfactory separation of the above compounds was obtained by this method. The thyroids were removed 24 hr after the administration of radioactive iodine and were hydrolysed with 2N NaOH for 16 hr at 100°C. Chromatography showed the presence of thyroxine, TIT, iodide, DIT, and MIT, and smaller amounts of a number of unidentified compounds. A fast-running substance appeared with the solvent front ahead of the thyroxine, and further unknowns appeared between thyroxine and TIT, between TIT and iodide, and between iodide and DIT. Analysis of such a hydrolysate gave the following figures: thyroxine 12·4, TIT 2·6, iodide 18, DIT 23·4, and MIT 11·4, all expressed as a percentage of the total radioactivity. No measurements of specific activity were made. A fraction which was washed out of the column with water after the MIT amounted to 13 per cent. and may be incompletely degraded material. Unknowns and intermediate background made up the remaining 19 per cent.

(*b*) *Free Iodo-amino Acids of the Thyroid*

When a solution of unhydrolysed thyroid was placed on the column a fast-running unknown appeared, followed by thyroxine, TIT, and iodide. After the emergence of the iodide the background level of radioactivity steadily increased with the DIT and MIT peaks clearly superimposed. These two fractions were run on paper with *n*-butanol–acetic acid and showed the presence of the two iodinated tyrosines, together with some material near the origin which may be peptide in nature. The major part of the radioactivity, presumably thyroglobulin, was washed out of the column with water.

In all animals examined, except one, the free thyroxine was less than the free TIT, from about one-half to one-fifth as much. Cold 2–3N NaOH would not be expected to produce extensive hydrolysis of thyroglobulin in the 6–8 hr which were required to run the thyroxine and TIT out of the column. The fact that the carrier amino acid and the radioactivity ran parallel provides evidence against the liberation of significant amounts of free thyroxine and TIT during the separation. The presence of labile linkages rapidly split on solution of the thyroglobulin in alkali cannot be excluded. Free DIT was always less than free MIT.

(c) *Comparison of Free and Combined Iodo-amino Acids*

The thyroids of four rats, removed 24 hr after receiving 25 μc each of ^{131}I, were dissolved in 0·5 ml of 2N NaOH. An 0·3 ml aliquot of this solution was run on the column for estimation of free thyroxine and TIT, and another 0·1 ml run after hydrolysis for 16 hr at 100°C with a further 0·1 ml 2N NaOH. The free thyroxine was 0·09 per cent. and the free TIT was 0·16 per cent. of the total ^{131}I. In the hydrolysed sample thyroxine was 2 per cent. and the TIT was 0·65 per cent. of the total ^{131}I.

(d) *Free Iodo-amino Acids of the Thyroid after Radiation Damage*

In one animal given 10 μg of thyroxine to inhibit thyroid secretion 24 hr before the administration of 300 μc of ^{131}I, and 5 μg of thyroxine daily thereafter, the thyroid radioactivity measured *in vivo* fell sharply after 48 hr. At 72 hr it had fallen to one-third of the 48 hr value, and at 96 hr it had fallen to one-third of the 72 hr value. At this stage the gland was removed and examined for free amino acids. A trace of thyroxine was found and no TIT. Iodide, DIT, and MIT were present, the amount of DIT being at least twice the MIT, as estimated from the area under the curve on the recorder.

(e) *Iodine Compounds of the Plasma*

When plasma was placed on the column, the radioactive thyroxine was usually spread more than when the pure substance alone was run. It appeared earlier, and finished later, but in all cases of spreading, added thyroxine carrier behaved like the radioactivity. The other substances behaved normally in the presence of plasma.

In two animals the distribution of radioactive compounds in the plasma was examined at 24-hr intervals after the administration of ^{131}I. The results are shown in Table 1.

In the animal which received 1 mc ^{131}I (Table 1), thyroglobulin was found at 24 hr and iodinated tyrosines at 72 hr. The latter were not estimated separately at this time. At 96 hr, DIT and MIT were 2·7 and 4·7 per cent. respectively of the total plasma iodine. The large amount of iodide present in the plasma of this animal was probably due to the fact that on this occasion the ^{131}I injected contained considerable amounts of ^{127}I. At this time the ^{131}I contained about 5 μg of ^{127}I per mc on the date of shipment, and the material had been kept in the laboratory for some time so that the dose injected might have contained as much as 40 μg of iodide, a small proportion only of which would be concentrated by the thyroid. In the animal which received

200 μc of ^{131}I (Table 1), thyroglobulin appeared at 96 hr. No iodinated tyrosines were found in the plasma of this rat. In neither animal was TIT found in the 0·2 ml of plasma used for direct chromatography.

A larger amount of plasma (2·3 ml) from the rat receiving 1 mc ^{131}I was obtained by pooling the residues of the daily samples of the first to the fifth days. The radioactivity was extracted by making the plasma twice normal with respect to sodium hydroxide, absorbing on 10 g of kieselguhr, and eluting with *n*-propanol. The alcoholic solution was concentrated under reduced pressure at room temperature and the concentrate run on a column after the addition of thyroxine and TIT carriers. A small radioactive peak, coincident with the TIT was found and amounted to 1·4 per cent of the thyroxine present or about 0·01 per cent. of the plasma ^{131}I. Similar treatment of 2·3 ml of pooled plasma from the animal receiving 200 μc showed no detectable radioactivity with the carrier TIT.

TABLE 1

DISTRIBUTION OF LABELLED COMPOUNDS IN PLASMA AFTER ^{131}I ADMINISTRATION

Dose	Time after ^{131}I Administration (hr)	Total ^{131}I in 0·2 ml Plasma (counts/min)	Components in 0·2 ml Plasma (counts/min)				Components as % of Total Plasma ^{131}I			
			Thyroxine	Iodide	DIT and MIT	Protein	Thyroxine	Iodide	DIT and MIT	Protein
1 mc	24	3650	163	3250	32	191	4·5	89·5	0·9	5·3
	48	3090	193	2665	16	191	6·3	86·9	0·5	6·2
	72	5660	365	2370	585	2530	6·2	40·5	10·0	43·2
	96	4712	352	2740	348	1260	7·5	58·3	7·4	26·8
200 μc	24	400	304	96	—	—	76	24	—	—
	48	400	228	172	—	—	57	43	—	—
	72	460	208	253	—	—	45	55	—	—
	96	640	205	282	—	153	32	44	—	24
	120	750	270	374	—	105	36	50	—	14

Reference has been made above to the examination of the free amino acids in the thyroid of a rat which received thyroxine injections and 300 μc of ^{131}I, and showed extensive loss of thyroid iodine after 48 hr due to radiation damage. Plasma from this animal taken at 72 hr showed the presence of considerable amounts of thyroxine, iodide, DIT, and thyroglobulin, together with traces of TIT, and MIT.

In a further experiment five rats were given 100 μc of ^{131}I each and were bled 24 hr later. One ml of the pooled plasma was extracted with *n*-butanol after being made alkaline with sodium hydroxide. The concentrated butanol extract on analysis showed the presence of thyroxine (62 per cent.), TIT (2·5 per cent.), iodide (28 per cent.) and combined MIT and DIT (8 per cent.).

IV. Discussion

The chromatographic system used in these investigations has certain advantages over most of the systems which have been used hitherto for this type of investigation. Small amounts of plasma can be applied directly to the column and the radioactivity can be quantitatively accounted for in the effluent. This obviates the incomplete recovery found when attempts are made to isolate the iodine compounds by extraction and concentration. However, this direct chromatography of the unextracted plasma is only possible when the specific activity is high. The examination of plasma with weak radioactivity which requires preliminary extraction of large volumes of plasma is less satisfactory, because spreading of the peaks is encountered.

Use of ultraviolet absorption of the fractions as indicator of the position of added carrier substances facilitates comparison of the distributions of the radioactive compounds and the carriers. The recovery of separate fractions from the column can be easily achieved by evaporation and is more satisfactory than the elution of spots from paper chromatograms.

The examination of the free amino acids of the thyroid shows that thyroxine, TIT, DIT, and MIT can be easily demonstrated in every case. It is striking that, in general, the amount of free TIT is in excess of the amount of free thyroxine and that the amount of free MIT is in excess of the amount of DIT. The relatively small amount of free thyroxine found in thyroid tissue is ascribed to the effect of selective secretion of thyroxine by the gland and the relative accumulation of free TIT is considered to indicate that this compound is secreted less readily than thyroxine. The relative preponderance of free MIT, as compared with free DIT, does not appear to be due to any secretion of DIT by the thyroid since we have not found any DIT in plasma except under conditions when severe radiation damage has caused a leakage of thyroid constituents, including thyroglobulin, into the plasma. Free DIT and MIT in the thyroid are presumably disposed of by de-iodination (Roche *et al.* 1953). It would appear that DIT is disposed of more rapidly than MIT.

From the results of alkaline hydrolysis of thyroid tissue, it is clear that TIT, like thyroxine, is present in thyroglobulin since the absolute amount of TIT increased during hydrolysis. The amount of TIT, however, in the thyroglobulin was considerably less than the amount of thyroxine, in contrast to the ratio found for the free amino acids.

Some comment is called for by the low percentage of thyroxine found in one of the thyroids examined after hydrolysis. We believe that this low value is associated with a low concentration of iodine in the gland. In some especially hyperplastic thyroid glands we have found less than 1 per cent. of the radioactivity present in the form of thyroxine after hydrolysis. Similar observations have been made on transplanted thyroid tumours in the rat in which the iodine content was much lower than that of normal thyroid tissue. The low iodine content of the thyroids may also be responsible for the relatively large amounts of MIT found. In the one animal treated with thyroxine, in which an increased thyroid iodine content would be expected, free DIT was found in excess of MIT.

Our investigations on the iodine compounds of plasma indicate that under normal conditions the major component is in the form of thyroxine. Iodide is always present. Both these substances are present in the plasma before radiation damage effects appear, as signalled by the appearance of thyroglobulin, MIT, and DIT in the plasma.

Since our animals were iodine-deficient and their thyroids contained easily demonstrable amounts of free TIT we considered it likely that they would be especially favourable subjects for the demonstration of TIT as a product of thyroid secretion. It also seemed reasonable to assume that in these animals the plasma level of TIT would be raised by radiation-induced leakage from the thyroid. Despite this, TIT could be found only with difficulty in some of the animals suffering from the effects of radiation damage and not at all in others. It has not yet been determined to what extent radiation-induced leakage from the thyroid gland is responsible for the TIT found in the plasma in these experiments. With ^{131}I doses which do not produce radiation damage, the total radioactivity level of the plasma is low, and more sensitive methods than we have used here for the measurement of radioactivity would be needed to detect traces of TIT under these conditions.*

V. Acknowledgment

We are indebted to Dr. B. A. Hems, Glaxo Laboratories Ltd., for a gift of thyroxine and di-iodothyronine.

VI. References

Barker, S. B. (1955).—The thyroid. In "Brookhaven Symposia in Biology." No. 7. p. 74. (Brookhaven Natl. Lab.: New York.)

Benua, R. S., and Dobyns, B. M. (1955).—*J. Clin. Endocrinol.* **15**: 118.

Gross, J. (1954).—*Brit. Med. Bull.* **10**: 218.

Kennedy, T. H. (1953).—*Proc. Univ. Otago Med. Sch.* **31**: 33.

Robbins, J., Peterman, M. L., and Rall, J. E. (1954).—*J. Biol. Chem.* **208**: 387.

Roche, J., and Michel, R. (1954).—*Annu. Rev. Biochem.* **23**: 481.

Roche, J., Michel, O., Michel, R., and Lissitsky, S. (1953).—*C. R. Soc. Biol., Paris* **147**: 232.

Tong, W., Taurog, A., and Chaikoff, I. L. (1952).—*J. Biol. Chem.* **195**: 407.

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* Since this paper was completed, TIT has been found in the plasma of rats given small non-damaging amounts of ^{131}I. The amount present was about 1 per cent. of the thyroxine.

RUMINAL FLORA STUDIES IN THE SHEEP

VI. DIURNAL, DAILY, AND SEASONAL FLUCTUATIONS IN THE CONCENTRATION OF "FREE" RUMEN BACTERIA AND IN RUMEN pH

By M. C. Nottle*

[*Manuscript received April* 9, 1956]

Summary

Diurnal, daily, and seasonal fluctuations in the concentrations of bacteria within the rumen and in rumen pH were determined in mature sheep.

Significant diurnal fluctuations were found in both bacterial concentrations and rumen pH, and marked differences occurred in the nature of the diurnal curve for bacteria in sheep which were fed two diets identical except for the protein source in the concentrate portion of the ration.

For each time of sampling (0, 3, and 6 hr after feeding) significant daily differences in rumen bacterial concentrations were observed.

In the seasonal variability trial, in which the sheep were fed a fairly constant diet for a period of nearly 2 years, a seasonal fluctuation in rumen bacterial concentration was found. The pH of the rumen was also found to fluctuate seasonally in a manner similar to the seasonal fluctuations in rumen bacterial concentrations. The minimum bacterial concentration and the minimum pH, which occurred in May, were significantly lower than the maxima which occurred in October.

The significance of these findings is discussed, and attention drawn to the similarity of the curves of hours of daylight and mean monthly temperatures and the seasonal curve of rumen bacterial concentration. The suggestion is made that light and temperature, as well as diet, may influence the seasonal fluctuation in rumen bacteria.

I. Introduction

The outstanding importance of the vast number of microorganisms in the rumen to the nutritional physiology of the sheep makes it imperative that the factors influencing the numbers and types of these organisms be thoroughly understood. True dietary effects can only be assessed if the fluctuations in rumen microbial population which occur under constant dietary conditions are known. To throw some light on this problem a series of experiments was undertaken with sheep fed single diets in which the diurnal, daily, and seasonal changes in the concentration of rumen bacteria were observed. Parallel observations on the changes in rumen pH were also made.

Several groups of workers have attempted to show that diurnal fluctuations in the concentration of rumen bacteria occur in sheep and cattle. Not only were inconsistent results obtained but different methods were employed. Thus Johnson *et al.* (1944) used plate counts, which were not specified as being anaerobic, and Bortree *et al.* (1946) and Chance *et al.* (1953) used the iodine staining reaction. The limitations of the plate counts are obvious, whilst the method used by the other workers, since the iodine staining reaction is dependent on the nutritional status of

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the bacteria, may or may not indicate fluctuations in concentration of the whole population. Henneberg (1922) has shown that the rumen population can be divided into iodophilic and non-iodophilic bacteria. Moir and Williams (1950) made direct counts of the "free" bacteria in rumen liquor after staining with aniline blue, and found that the concentrations obtained by this means agreed well with those obtained by the nigrosine smear method of Gall, Stark, and Loosli (1947). This latter method, rather than more specific methods, was chosen for the present study as it seemed to offer a better assessment of the ability of different diets to support a bacterial population.

Marked seasonal fluctuations in rumen bacterial concentration were observed in grazing sheep by van der Wath and Myburgh (1941) and Moir (1951). These fluctuations were attributed to seasonal changes in the nutritional quality of the herbage grazed. The possibility of such seasonal fluctuations occurring in sheep or cattle consuming diets of relatively constant composition has not previously been investigated, so far as is known.

II. Experimental

The investigations undertaken, together with diets, methods of feeding, numbers of animals in each determination, sampling times, and the observations made at each sampling time are presented in Table 1.

(a) *Design*

In each experiment a randomized-block design was used and the significance of all results, except feed and water consumption in the diurnal and daily experiments, was tested by means of the analysis of variance.

(b) *Animals*

Mature Merino wethers of similar age, weight, and appearance were selected at random from a large group and allocated to each of the experiments, except that, as far as possible, the same sheep were used in the second series of experiments on diurnal variations as were used for these experiments in the previous year.

(c) *Diets*

The daily diet used in the diurnal variation experiments consisted of 550 g oaten chaff plus 183 g of a concentrate mixture having the following composition: cracked wheat 82·5 g, wheaten starch 55·0 g, casein or wheat gluten 45·1 g, molasses 30 g, calcium carbonate 3·65 g and sodium chloride 3·65 g. In the first experiment the diet contained gluten and in the second casein, so that the two diets were identical except for the main protein source.

All weighings of the dietary components were made on the air dry material with the exception of the molasses which was weighed as the liquid. The concentrate materials were mixed in a concrete mixer, sprayed with a solution of molasses, and the mixture dried in a current of air at 55°C. The chaff and concentrate portions of the daily diet were weighed into a single bag and fed together in the first experiment

(1950) and into separate bags and fed simultaneously in separate feed boxes in the second experiment, as indicated in Table 1. The percentage crude protein in the gluten and casein diets were 9·4 and 10·4 per cent. respectively.

TABLE 1

EXPERIMENTAL DETAILS

Variability Tested	No. of Sheep	Diet	Method of Feeding Diet	Sampling Times	Observations
Diurnal	6	Chaff and gluten concentrate	Fed together	0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 20, and 24 hr after feeding	Rumen bacterial concentrations, feed, and water consumption
Diurnal	6	Chaff and casein concentrate	Fed separately	0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 20, and 24 hr after feeding	Rumen bacterial concentrations and pH, feed, and water consumption
Daily	5	Chaff and casein concentrate	Fed separately	0, 3, and 6 hr after feeding on each of 6 consecutive days	Rumen bacterial concentration, feed, and water consumption
Seasonal	4	Chaff and casein concentrate	Fed together	0, 3, and 6 hr after feeding on each of 2 successive days on eight occasions at approximately 3-monthly intervals over a period of 652 days	Rumen bacterial concentration and pH, feed, and water consumption

In the daily variability trial the casein diet was fed as just described. In the prolonged seasonal variability experiment the casein diet described above was used throughout, but the chaff and concentrate portions were fed together except during the rumen sampling of period 1, when each portion was fed in a separate feed box. Owing to storage limitations, mixing of concentrate at approximately 3-monthly intervals was necessary. This, together with unavoidable changes in the source of the chaff, resulted in some variability in the chemical composition of the rations as fed (Table 2). The crude fibre content was remarkably constant throughout but the protein content varied appreciably. The significance of this variation in composition is assessed below.

A progressive decline in feed consumption which was observed prior to period 3 suggested the possibility of cobalt deficiency. Analyses revealed that the diet was extremely low in cobalt and contained suboptimal amounts of copper. Both elements were incorporated in a drench and given for the first time on the 235th day of the experiment, i.e. 11 days after the completion of period 3. This was followed by a reduction in feed consumption which did not occur with later drenches containing cobalt only. Subsequently, adequate copper and cobalt were added to the diets.

(*d*) *Feeding Regime*

In each experiment the animals were fed daily at 9 a.m. At each rumen sampling period any feed residues were weighed and returned to the feed boxes after each sample had been taken. Adequate supplies of tap water were available to all sheep at all times, and in the seasonal variation experiment each sheep was drenched once a week with an oil rich in vitamins A and D except when this was within 7 days of rumen sampling.

Table 2

COMPOSITION OF THE RATIONS AS FED IN SEASONAL VARIABILITY TRIAL

Period	Dry Matter (g)	Crude Fibre (g)	Nitrogen (g)	Crude Protein in Dry Matter (%)
1	673	139	11·2	10·4
2	651	142	11·5	11·0
3	664	144	12·6	11·9
4	657	144	11·5	10·9
5	666	137	9·2	8·7
6	662	144	10·0	9·4
7	662	144	9·5	9·0
8	667	147	8·3	7·8

(*e*) *Sampling and Treatment of Rumen Material*

Rumen samples were obtained by stomach tube and pump (Moir and Williams 1950). An aliquot was formalized quantitatively for bacteriological study, and, where pH was determined, it was measured by means of a glass electrode immediately after the withdrawal of the sample. The nigrosine smear method of counting, as described by Moir (1951) and discussed by Williams and Moir (1951) was used. This enables counts to be made of all the "free" bacteria in the sample greater than 0·5 μ in diameter.

To determine diurnal variability, rumen samples were taken from each of six sheep in two trials at 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 20, and 24 hr after feeding (Table 1). Daily variability was assessed by taking rumen samples at 0, 3, and 6 hr after feeding from five sheep on each of 6 consecutive days. In the seasonal variability trial, rumen samples were taken from each of four sheep on 2 consecutive days, at 0, 3, and 6 hr after feeding, on eight occasions spaced at approximately 3-monthly intervals throughout the 655 days experimental period. The first sampling coincided with the last day of the eight 10-day "collection" periods when digestibility and nitrogen balance determinations were made on each sheep.

III. Results

(*a*) *Diurnal Fluctuations*

The mean results for rumen bacteria and pH are presented in Figure 1.

(i) *Rumen Bacteria.*—For both the gluten and casein diets there were significant period (time after feeding) differences ($P<0\cdot001$) in the concentration of free rumen bacteria. Animal differences were also highly significant for the gluten diet ($P<0\cdot001$) and significant at the $P<0\cdot05$ level for the casein diet.

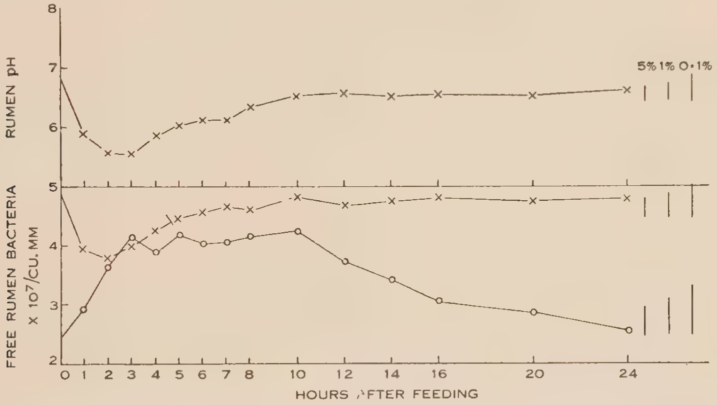

Fig. 1.—Diurnal fluctuations in free rumen bacterial concentration and rumen pH. ○ Gluten diet. × Casein diet. Least significant differences at the 5 per cent., 1 per cent., and 0·1 per cent. levels are indicated on the figure.

The diurnal variation curves were completely different for the two diets. With the gluten diet, the mean minimum bacterial concentration of 24 millions/cu.mm occurred immediately before feeding and rose to a maximum of 41 millions/cu.mm by 3 hr after feeding. The concentration remained at this level till 10 hr and thereafter slowly declined to pre-feeding level by 24 hr. The increase between 0 and 3 hr was highly significant ($P<0\cdot001$) but the 3–10 hr differences were not significant.

With the casein diet there was a sudden and highly significant ($P<0\cdot001$) decline from a maximum of 49 millions/cu.mm immediately prior to feeding to a minimum of 38 millions/cu.mm at 2 hr after feeding. Thereafter the concentration increased to pre-feeding level which was attained at about 10 hr. The differences between periods after 6 hr were, however, not significant.

(ii) *Rumen pH.*—No rumen pH determinations were made on the sheep receiving the gluten diet. On the casein diet period differences were highly significant ($P<0\cdot001$) but animal differences were not significant. It is apparent from Figure 1 that the diurnal trend in rumen pH was very similar to that of the rumen bacteria concentrations. Thus the maximum, pH 6·87, occurred at 0 hr followed by a significantly lower ($P<0{\cdot}001$) minimum of 5·53 at 3 hr. Thereafter the pH slowly increased but the differences between periods after 10 hr were not significant. All periods were significantly less than at 0 hr ($P<0{\cdot}05$).

(iii) *Feed and Water Consumption.*—Wide variability was observed between animals in the rate of both feed and water consumption but consumption was generally faster and more uniform in the casein than in the gluten experiment. This is illustrated by the following average figures: an average of 361 g feed and 308 ml water were consumed in the first hour after feeding on the gluten diet, whereas on the casein diet an average of 569 g feed and 292 ml water were consumed in this period. By 3 hr after feeding the corresponding figures were 435 g and 441 ml, 617 g and 470 ml, respectively.

(*b*) *Daily Fluctuations*

The results for bacteria are presented in Figures 2 and 3.

(i) *Rumen Bacteria.*—Analysis of these data revealed that the following relationships were significant—days ($P<0{\cdot}05$), sheep ($P<0{\cdot}001$), time (after feeding) ($P<0{\cdot}001$), sheep × day interactions ($P<0{\cdot}001$), and day × time interactions ($P<0{\cdot}05$).

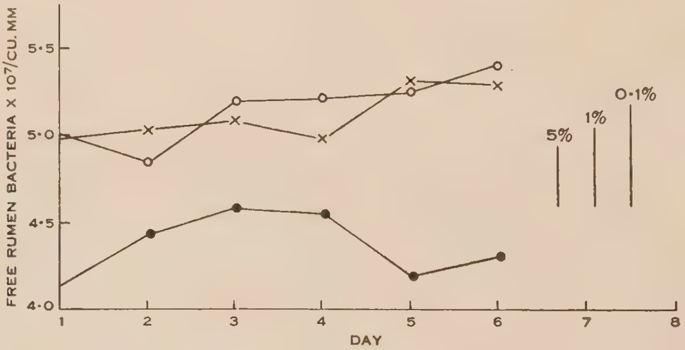

Fig. 2.—Daily variation in rumen bacterial concentration at three sampling times (time × day interaction). Each point is the mean for five sheep. ○ 0 hr, ● 3 hr, × 6 hr after feeding. Least significant differences at the 5 per cent., 1 per cent., and 0·1 per cent. levels are indicated on the figure.

It is apparent from Figure 2 that for each time of sampling (0, 3, and 6 hr after feeding) there are significant differences between days, but the days when these differences occur may differ between sampling times. It is evident also that the differences between sampling times are in agreement with those obtained with the casein diet in the diurnal variation experiment reported above.

The sheep × day interactions shown in Figure 3 indicate that while some sheep varied significantly in their daily means, others were relatively constant over the 6 days. The significance of the difference in means for individual sheep on any day also varied. Thus on the second day these differences were highly significant ($P<0{\cdot}001$) while on the fifth day they were not significant.

(ii) *Feed and Water Consumption.*—The average consumption of feed in the first 3 hr after feeding was 645 g, a total very close to that which occurred with this diet in the diurnal variation study. Water consumption, however, was much higher, averaging 1119 ml for the 3 hr.

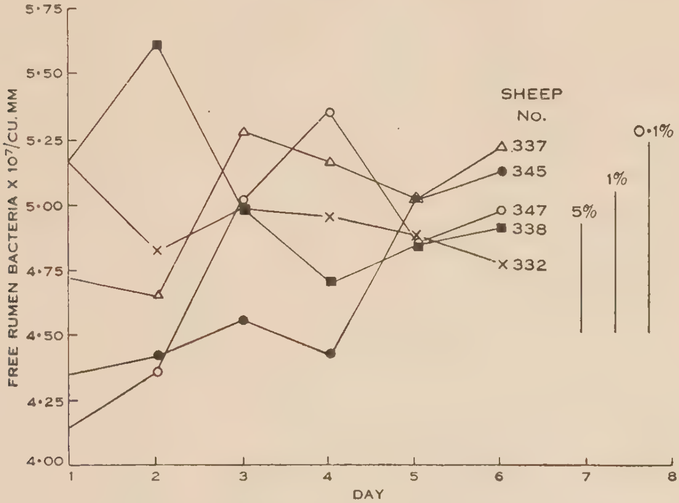

Fig. 3.—Daily variation in the rumen bacterial concentration for individual sheep (day × sheep interaction). Each point is the mean of three sampling times. Least significant differences at the 5 per cent., 1 per cent., and 0·1 per cent. levels are indicated on the figure.

(c) *Seasonal Fluctuations*

The mean results for all determinations, together with the least significant differences between any two periods, are given in Table 3.

In order to achieve statistical balance, the results for one sheep, which died from enterotoxaemia shortly after the fifth metabolism period, and the 3-hr values for rumen bacteria and rumen pH were omitted from the analyses. This was necessary because the 3-hr values were not available for periods 2 and 3. The results for rumen bacteria and pH therefore represent the mean of four sheep sampled on 2 consecutive days at 0 and 6 hr after feeding, while those for feed and water consumption are the mean amounts consumed over 6 hr on 2 consecutive days in each period. Sampling at these times, rather than at 0 hr only, was carried out in order to obtain some information on the nature of the diurnal pattern at different times of the year.

(i) *Feed and Water Consumption.*—With the exception of period 3, all sheep consumed the whole ration, so that the dry matter and nitrogen intakes are as given in Table 2. During period 3 a marked reduction in appetite and rate of food consumption occurred in three of the five animals. With the addition of cobalt both the rate and the total amount of food consumed increased so that the whole of the ration was consumed in subsequent periods. Differences in rate and amount of feed consumption between periods (other than period 3) were not significant. Differences between sheep

TABLE 3

MEAN EXPERIMENTAL RESULTS FOR THE SEASONAL VARIABILITY EXPERIMENT

Period number	1	2	3	4	5	6	7	8	Least Difference between Periods for Significance at		
Date	18.vi.51	24.ix.51	6.xii.51	12.ii.52	20.v.52	15.x.52	15.xii.52	9.ii.53			
Days on diet at commencement of determination	52	150	223	291	389	537	598	654	$P<0\cdot05$	$P<0\cdot01$	$P<0\cdot001$
Rumen bacteria (millions/cu.mm)	51·0	59·6	62·4	53·0	46·2	64·2	63·2	51·6	3·8	5·2	7·1
Rumen pH	—	6·9	6·6	6·7	6·3	6·8	6·5	6·6	0·15	0·21	0·29
Feed consumed in 6 hr (g)	724	643	279	733	683	733	665	729	169	230	310
Water consumed in 6 hr (ml)	1330	1150	863	1875	1050	1330	1236	1609	328	447	603

were also not significant, suggesting that the eating habits of the sheep were very similar.

Period differences in the rate of water consumption were significant ($P<0\cdot001$) but differences between animals and days were not significant.

(ii) *Rumen Bacteria*.—The concentrations of rumen bacteria differed significantly between periods ($P<0\cdot001$) and between times after feeding ($P<0\cdot001$). There were, however, no significant differences between days within periods, or between sheep, and the interactions period × time after feeding and days × time were not significant. The diurnal trend was similar to that reported earlier for the same ration.

Inspection of Table 3 indicates that there is a seasonal fluctuation in bacterial concentration. Covariance analysis showed that these highly significant period differences were not due to variations in the percentage protein in the diet. The following regression equation, which has no significant deviations, accounts for practically all period differences:

$$Y = 55\cdot0034+8\cdot7087 \sin(0\cdot9863(x-212))^\circ,$$

where Y is bacterial concentration in millions/cu.mm and x is the number of days after January 1st. The equation indicates that the bacterial concentration fluctuates sinusoidally about a mean of 55·0 millions/cu.mm and that the minimum and maximum concentrations occur on the 121st and the 303rd day of the year, i.e. on May 1st and October 30th. At these times they are 46·3 and 63·7 millions/cu.mm, respectively. This seasonal trend is similar to that reported for grazing sheep by van der Wath and Myburgh (1941) and by Moir (1951), but the seasonal fluctuations are much smaller, due in all probability to the much more constant composition of the diet in the present experiments.

Qualitative examination of the bacterial population in each period was not made, but it was evident from the nigrosine smears that the appearance of the population in period 3 differed from that of all other periods. The most striking feature of this period was the appearance of large forms, and of *Oscillospira* species in one animal, whereas in previous and subsequent periods the population consisted mainly of small forms. If cobalt deficiency was responsible for these changes, the effects contrast greatly with those of Gall *et al.* (1949) who observed a considerable reduction in bacterial concentration in cobalt deficiency but only slight qualitative changes.

(iii) *Rumen pH*.—The results presented in Table 3 indicate that the pH of the rumen fluctuates seasonally, in a manner similar to the seasonal fluctuations in rumen bacterial numbers. The minimum, which occurred in May, is significantly lower than the maximum which occurred in September and October. In addition to these significant period differences, sheep and days within period differences were also significant ($P<0\cdot05$).

The diurnal trend throughout the experiment was the same as that reported earlier in the diurnal experiment.

IV. Discussion

The very different diurnal curves of rumen bacterial concentration which occurred in the sheep on the gluten and the casein diets, in which only one component of the whole diet was varied, indicates that comparisons between bacterial concentrations of animals on different diets should only be made when the nature of the diurnal curve on each diet is known. The finding that the greatest changes in bacterial concentration occur immediately after feeding indicates further that rumen samples should be taken as distant as possible from the immediate influence of feeding. With one daily feeding this, of course, means at 24 hr.

The greater percentage protein in the casein diet combined with its greater physical if not chemical availability, would be expected to favour a greater bacterial proliferation. No such effect is evident. On the other hand, the dilution of rumen contents by feed and water would presumably be larger on casein than gluten. The absence of evidence for any dilution on gluten suggests that other factors are involved in determining the diurnal curve. One possibility is the difference in the physical properties of the two concentrates. Whereas gluten enmeshed all other components of the concentrate, and the "nuts", when immersed in water, became swollen, glutenous, and retained their shape, the components of the casein concentrate were only slightly bound together by casein and the nuts disintegrated completely in water.

What these changes in concentration mean in terms of the total number of bacteria in the rumen at any time is unknown, but it is apparent that a method of determining rumen volume is required so as to take into account variations in volume which occur through feed and water consumption, saliva inflow, the passage of digesta from the rumen, and the passage of water into or out of the rumen through the rumen wall.

The diurnal fluctuations in rumen pH confirm the findings of several groups of workers (Monroe and Perkins 1939; Phillipson 1942; Myburgh and Quin 1943; and Chance *et al.* 1953) that there is a fall in rumen pH after the ingestion of food. The rate at which this fall occurs has been shown to be related to the availability of the substrate to the rumen bacteria (Phillipson and McAnally 1942). This factor, combined with the rapid consumption of the concentrates, probably accounts for the greater rate and extent of the pH changes in these experiments than in those of the authors cited above.

The small but significant day to day variations in rumen bacterial concentrations found in sheep receiving the same diet fed in the same way conflict somewhat with the findings of Moir and Williams (1950) and Williams and Moir (1951). These workers obtained excellent agreement between days when the same sheep were sampled at the same time on each day. The random nature of the small daily variations in the present experiment suggests that they were largely a reflection of daily differences in the manner and rate of the animal's eating and drinking.

The seasonal fluctuations in rumen bacterial concentration which occurred under relatively constant dietary conditions are of great interest, especially as very similar seasonal trends were obtained by van der Wath and Myburgh (1941) and Moir (1951) where the sheep were exposed to marked seasonal differences in the quantity and quality of the herbage grazed. The dietary conditions in the present experiments, although highly constant compared with those in the earlier work just cited, were subject to some variations, mainly in crude protein content, due to the use of different batches of chaff and concentrate. The seasonal fluctuations in rumen bacteria which occurred could not be related to any such changes in composition of the ration. It seems therefore that some factor or factors other than diet were responsible for the seasonal changes in rumen bacterial concentration.

In Figure 4 the curves of hours of daylight (sunrise to sunset) and mean monthly temperatures are plotted with the seasonal curves of rumen bacteria. There is good agreement between all three curves, especially between daylight and bacteria which are both sine curves. This suggests that light and temperature may influence the seasonal fluctuation in bacteria. Light and temperature are believed to affect reproductive functions through their influence on the pituitary and thyroid glands, and light is known to affect coat changes in cattle and wool growth in sheep. The rumen environment could be influenced either by a direct effect on rumen temperature, which seems extremely unlikely in the present experiments, or by an effect on blood composition. Alterations in blood composition would presumably be reflected in saliva composition and the interchange of ions and water between rumen liquor and blood (Parthasarathy and Phillipson 1953). It is possible that fluctuations in rumen bacteria result from corresponding fluctuations in some substance or substances entering the rumen via the saliva or the rumen wall or leaving the rumen through the rumen wall.

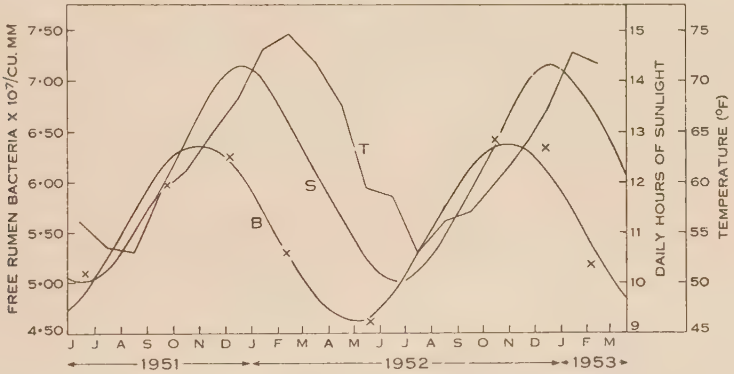

Fig. 4.—Seasonal fluctuations in rumen bacterial concentration (*B*), hours of sunlight (sunrise to sunset) (*S*), and mean monthly temperature (*T*). × Experimental values.

Digestion and nitrogen metabolism experiments, to be reported elsewhere, were conducted concurrently with the seasonal bacterial determinations but, although there were significant period differences in the digestion of crude fibre, dry matter and nitrogen, and in nitrogen balances, these could not be related to fluctuations in the concentration of rumen bacteria.

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VI. REFERENCES

BORTREE, A. L., DUNN, K. M., ELY, R. E., and HUFFMAN, C. F. (1946).—*J. Dairy Sci.* **29**: 542.

CHANCE, C. M., SMITH, C. K., HUFFMAN, C. F., and DUNCAN, C. W. (1953).—*J. Dairy Sci.* **36**: 743.

GALL, LORRAINE S., SMITH, S. E., BECKER, D. E., STARK, C. N., and LOOSLI, J. K. (1949).—*Science* **109**: 468.

GALL, LORRAINE S., STARK, C. N., and LOOSLI, J. K. (1947).—*J. Dairy Sci.* **30**: 891.

HENNEBERG, W. (1922).—*Zbl. Bakt.* II Abt. **55**: 242.

JOHNSON, B. C., HAMILTON, T. C., ROBINSON, W. B., and GEORGE, J. C. (1944).—*J. Anim. Sci.* **3**: 287.

MOIR, R. J. (1951).—*Aust. J. Agric. Res.* **2**: 322.

MOIR, R. J., and WILLIAMS, V. J. (1950).—*Aust. J. Sci. Res.* B **3**: 381.

MONROE, C. F., and PERKINS, A. E. (1939).—*J. Dairy Sci.* **22**: 983.

MYBURGH, S. J., and QUIN, J. I. (1943).—*Onderstepoort J. Vet. Sci.* **18**: 119.

PARTHASARATHY, D., and PHILLIPSON, A. T. (1953).—*J. Physiol.* **121**: 452.

PHILLIPSON, A. T. (1942).—*J. Exp. Biol.* **19**: 186.

PHILLIPSON, A. T., and MCANALLY, RACHEL A. (1942).—*J. Exp. Biol.* **19**: 199.

VAN DER WATH, J. G., and MYBURGH, S. J. (1941).—*Onderstepoort J. Vet. Sci.* **17**: 61.

WILLIAMS, V. J., and MOIR, R. J. (1951).—*Aust J. Sci. Res.* B **4**: 377.

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